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ON THE CULTIVATION OF *TRYPANOSOMA BRUCEI*.*

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IN our paper on the cultivation of *Trypanosoma lewisi*¹ we showed that this parasitic protozoön could be readily cultivated outside of the living body. At that time we expressed the belief that the same method would in all probability be applicable to other trypanosomes. This view has been confirmed apparently by Rabinowitsch and Kempner,² who, in a footnote, briefly state that the method is suitable for the cultivation of the trypanosome of Caderas. In a recent preliminary note³ a brief summary was given of our work on *Trypanosoma brucei*, and the purpose of this paper is to present the details of that investigation.

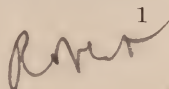
Our knowledge of this interesting parasite dates back only to 1895, in which year Bruce published a preliminary report on the results of his study of Nagana, or the tsetse-fly disease of Zululand. A more extended report was published by him in

* Received for publication November 18, 1903.

¹ *Contributions to Medical Research*, dedicated to Victor Clarence Vaughan, Ann Arbor, Mich., 1903, George Wahr, p. 549.

² *Centralbl. f. Bacteriol. (Orig.)* 1903, 34, 816.

³ *J. Am. Med. Assoc.*, November 21, 1903.

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1897. It was clearly shown from these investigations that the disease was due to a flagellated protozoön similar to that previously described by Evans as existing in the Surra of India. This organism was subsequently designated by Plimmer and Bradford as *Trypanosoma brucei*. Bruce further demonstrated that the disease was spread by the bite of infected tsetse flies. The trypanosomes were found alive in the insects' proboscides up to forty-six hours after the flies had sucked infected blood. Motile parasites were still found in the flies' stomachs after 118 hours, but after 140 hours their stomachs were empty, and apparently dead parasites were found in the excreta. When contaminated flies were kept for twelve to forty-eight hours before they were allowed to bite healthy dogs, these only sickened on the thirty-second to thirty-eighth day, instead of after two weeks, as is usually the case. Nagana blood when removed aseptically produced the disease when kept for four days, but not after seven days. The blood when dried on threads produced infection after twenty-four hours in only one of three animals.

Kanthack, Durham, and Blandford in 1898 published the results of their studies on the trypanosome of Nagana. The material for this study was obtained from the blood of an infected dog brought to England from Zululand in 1896. We may say in passing that all of the subsequent investigations made in Europe, except that of Martini, were made with trypanosomes derived from this source. The observers mentioned found that in drawn blood, or in serous fluids, the hematozoa soon became motionless. This may occur rapidly, for instance in twenty minutes, but generally some motile specimens may be found after two to three days; sometimes, indeed, after as long as five or six days. Within a cadaver the blood and organs became non-infective in about twenty-four hours. They made numerous attempts at cultivation in normal blood, but while they observed the formation of tangles or agglutination masses, and, eventually, degeneration forms represented by masses of spherules, they failed to obtain any evidence of multiplication.

Plimmer and Bradford, in 1899, likewise noted the rapid disappearance of the trypanosomes from the blood after death, and

in a measure ascribed this to the rapid onset of decomposition. While the blood in the dead body may lose its infectivity in a few hours after death, they found that a large quantity of blood (200 c.c.) kept in a sterile flask, in an atmosphere of oxygen, was infectious for at least three days. Under the cover-glass, desiccation being prevented by means of a paraffin ring, they were able to observe motile trypanosomes for six days after removal from the body. They found that an addition to the blood of one-tenth its volume of a 5 per cent. solution of sodium citrate served to prolong the vitality of the trypanosomes.

In their admirable study of the Nagana trypanosome, Laveran and Mesnil (1902) showed that a similar result is attained when the trypanosome blood is diluted with serum. In a mixture of equal volumes of defibrinated Nagana rat blood and horse serum the organisms were motile for three days, whereas they had disappeared from the undiluted blood in that time. They also noticed that the serum of refractory animals, such as birds and man, was as serviceable as that from susceptible animals.

On the other hand, human serum, unlike all other sera, was found to exert a specific action upon the trypanosomes of Nagana in infected mice and rats. The subcutaneous injection of $\frac{1}{2}$ to 1 c.c. of the human serum into these animals, at the time when the trypanosomes are just beginning to appear in the blood, causes the parasites to disappear in from twenty-four to thirty-six hours. The blood then remains free from the organisms for four to eight days, exceptionally for twelve, eighteen, or nineteen days, after which they reappear. On subsequent injection of serum they again disappear for a short time. In this way the life of the animal may be prolonged, as with injections of arsenite of soda, but eventually death results. In four instances, however, one or two injections of the serum in mice were followed by recovery. More recently (July, 1903) Laveran showed that human serum exerts a similar action upon the trypanosomes of Caderas and Surra.

Laveran and Mesnil studied the action of heat and cold upon the Nagana trypanosomes, and their results may be briefly summarized. Nagana blood which has been heated for three hours at 40° infects mice, but the period of incubation (five to six days)

is a trifle longer than that observed when unheated blood is used. When the blood was heated for one hour and twenty minutes at 42° , the period of incubation was prolonged to eight days; that which had been heated at 44.5° was no longer infective. From this it is evident that a temperature of $40-42^{\circ}$ kills the trypanosomes slowly, while that of 45° is rapidly fatal.

The statement was made in our first paper that Laveran and Mesnil found the trypanosomes alive after an exposure of one-half to two hours at -50 to -55° . The blood in question was first exposed for half an hour to -15° , and then for five minutes to -50 to -55° , after which, on allowing it to thaw out for two hours, the trypanosomes were found to be motile and infectious. From a private communication of Dr. Mesnil we learn that he has found the Nagana and Caderas trypanosomes to resist the temperature of liquid air (-191°) for a quarter of an hour.

Like the previous observers, Laveran and Mesnil found that Nagana blood when left *in vitro* may lose its virulence in forty-eight, or even in twenty-four hours. When mixed with physiological salt solution the trypanosome may be alive for three days, but the result is not constant. Even when the blood is kept in the ice-box at $+5$ to 7°C. the virulence is lost in from three to five days, the same as when kept at the room temperature. It is worthy of note that they found such non-virulent blood to contain, at times, slightly motile trypanosomes. We will have occasion to show that living cultures of *Trypanosoma brucei* may be wholly non-virulent.

From the foregoing summary it will be noted that no one has been able to keep the *Trypanosoma brucei* alive for more than six days, and at no time has any evidence of multiplication *in vitro* been observed. Moreover, the virulence of the blood rapidly disappears, and only exceptionally is found to persist up to the fourth day.

The immediate purpose of our investigation was to demonstrate that the Nagana trypanosome, notwithstanding its apparently obligative parasitic character, could be cultivated *in vitro* as in the case of the rat trypanosome. We were able to undertake

this work through the kindness of Dr. H. Wolferstan Thomas, late Fellow at McGill Medical College and now of the Liverpool School of Tropical Medicine, who with extreme cordiality supplied us with the infected blood. It gives us very great pleasure to thus acknowledge his valuable help, without which it would not have been possible to arrive at our results as early as we have. Our Nagana material is derived from that brought to England by Dr. Waghorn in 1896, and is therefore the same as that employed by the English and French investigators.

The methods which we have employed in this work are essentially the same as those described in our first paper, and for that reason need no detailed consideration at the present time. It may be stated that the sealing of culture tubes and flasks has been effected by means of thick rubber caps, which are more convenient than the sealing wax heretofore employed.

The culture medium usually employed in our work was ordinary nutrient agar containing variable amounts of defibrinated or laked rabbit blood. This medium by no means constitutes a perfect soil for the growth of *Trypanosoma brucei*, as will presently be seen, for the simple reason that we do not understand as yet the exact conditions which must be supplied in order to secure multiplication. A study of these factors is at present engaging our attention. Flask cultures are more successful than those in tubes, partly because of the thin layer of fluid, and partly because of the abundant supply of oxygen. The flasks were inoculated by means of capillary pipettes; the tubes usually with a platinum wire with 2 mm. loop.

The fact that this organism may appear in the blood of an animal in enormous numbers, and, within a few hours after death, may disappear entirely indicates an extreme sensitiveness to some as yet unknown substance which, presumably, forms in the blood as a result of post-mortem change. The living blood may be considered as a physiologically balanced salt solution with reference to the sensitive trypanosome. Upon the death of the blood this equilibrium is destroyed as a result of the cleavage changes which take place. The toxic action of the salts or ions thus formed must be neutralized before the trypanosomes can be

made to grow. The decrease in the oxygen contents of the blood in the cadaver undoubtedly constitutes an additional factor in the destruction of trypanosomes.

It may be assumed that the blood remaining in the cadaver acquires a greater amount of the toxic compounds from the surrounding tissues than is the case when the blood is at once transferred to a tube. This, together with the fact that the oxygen contents are decreased, explains the more rapid death of the trypanosomes in the cadaver as compared with the test-tube. We have had no difficulty in keeping defibrinated Nagana rat blood in the test-tube, in the presence of oxygen for seven days.

The rat trypanosome is far less sensitive to the post-mortem alterations in the blood, and, for that reason, it may be kept alive in defibrinated blood for weeks and even for months, and, moreover, as will be seen, it will grow well on our culture medium when the Nagana parasite fails entirely.

The result of our first experiment, while not exactly successful, served to assure us that cultivation was possible. The blood of a white mouse, extremely rich in trypanosomes, was planted on a medium consisting of equal parts of defibrinated rabbit blood and ordinary agar. Examinations, made from day to day, showed that the organisms originally planted gradually decreased, till on the eighteenth day only one tube was found to contain a few motile trypanosomes. On this day the liquid contents of the tube were injected into a white rat, but no infection followed, showing that the organisms had become too enfeebled. Moreover, transplants made to fresh media failed to develop. The trypanosomes in the original tube showed more or less marked morphological alteration, appearing as spherical or kite-shaped forms, or as masses of small, round, granular bodies representing the débris of agglutination clumps or tangles, and only occasionally were typical organisms met with.

The significant feature of this experiment was the fact that the trypanosomes were kept alive for eighteen days, or three times longer than had been observed by others.

Notwithstanding numerous modifications of our technic, all our attempts to obtain cultures from the first twenty-five animals

failed. In the inoculated tubes the trypanosomes would usually die out on about the fifth to the seventh day; exceptionally they were found to be still alive on the ninth, tenth, eleventh, fourteenth and eighteenth day. The trypanosomes which were placed in the incubator usually died out in two or three days.

The culture media were constantly checked by inoculating control tubes with *Trypanosoma lewisi*, which invariably gave an abundant growth. Clearly, the Nagana trypanosome required other conditions than did the rat parasite. This fact was brought out early, and in a most unexpected manner, as will be seen from the following:

Owing to a scarcity of new white rats, it became necessary to inoculate a rat which had previously been injected with *Tr. lewisi* but had entirely recovered. At least, repeated examinations of the blood failed to show any parasites. Four days after the inoculation with Nagana the rat died and the cadaver was placed on ice over night. In the morning, the blood, which showed about one organism in each field of the microscope, was planted into several tubes of blood agar.

In the course of a week excellent growths of a trypanosome were obtained, and at first it seemed as if the cultivation of *Trypanosoma brucei* was realized. In appearance the organisms corresponded exactly with the forms observed in cultures of *Trypanosoma lewisi*. This fact, together with the uniform failure at that time to duplicate this result from other Nagana animals, and the unsuccessful inoculation of mice and guinea pigs, soon convinced us that the culture obtained was one of *Tr. lewisi* and not of *Tr. brucei*.

This experiment was repeated with like result. A wild rat infected with *Tr. lewisi* was inoculated with Nagana. Both parasites appeared in the blood and could easily be recognized during life. After death the blood was transferred to culture tubes, and, as in the previous case, in the course of a week or two abundant growths of *Tr. lewisi* were obtained, but no sign of the Nagana parasite could be detected.

Apart from the differences in the morphological characteristics of the pure cultures of the two trypanosomes, which, as will be

presently pointed out, are quite considerable, we have in the fact just given a ready means of differentiating the two organisms. A somewhat similar procedure was suggested by Koch,⁴ who found that when the rat blood which contained the two trypanosomes was injected into a dog the latter died of Surra (Nagana?). It is evident that by combining these two methods pure cultures of these organisms *in vitro* can be obtained. Thus the blood showing double infection can be inoculated into blood agar (1:2) and also into a mouse, guinea pig, or dog. The blood-agar tubes will give a pure culture of *Tr. lewisi* since the Nagana trypanosome will not grow on this medium; while from the animal, after death, the *Tr. brucei* can be isolated by inoculating blood agar tubes (2:1).

From the second series of twenty-five Nagana rats, mice, and guinea pigs we succeeded in obtaining four positive results. These cultures or strains, obtained from four different animals, we will designate as A, B, C, and D.

CULTURE A.

Generation I.—This was started on August 27. The blood was drawn from the living heart of a white mouse, on the fifth day after inoculation, at which time it was literally swarming with the parasites. Six tubes of blood agar (2:3) were inoculated and set aside at the room temperature. As is usually the case, the trypanosomes originally planted gradually decreased in number. Involution forms, by which are meant spherical or kite-shaped individuals, motile or dead, as well as masses of small round bodies, the remnants of degenerated trypanosomes, were present.

On September 12 (sixteenth day), in only one tube out of the six, a few motile, typical cells were to be found, and that in a whole cover-glass preparation. Four days later several small masses composed of eight or ten actively motile cells were met with. The appearance and motion of these organisms were such as to justify the belief that they were not mere survivals of the original trypanosomes planted, but rather new individuals developed from these by multiplication. On September 18 (twenty-second day) there was no longer any doubt. The rosettes or

⁴ *Reiseberichte*, 1898, 71.

colonies of actively motile trypanosomes had markedly increased in number and in size. The individual cells were vigorously active, and, in this respect, wholly unlike the slowly dying forms heretofore observed. The trypanosomes remained alive in this tube until after October 10, that is to say, for at least forty-four days.

Of the five tubes which were negative on September 12 one gave evidence of growth on the 16th. From that time the number of trypanosomes increased for about ten days, after which they began to die out, though some living cells were still present on the forty-fourth day.

Generation II.—This was made on September 18 from the first tube of the preceding generation. In order to insure sufficient inoculation the transplantation was made by means of a capillary tube pipette. Three of the six tubes of blood agar thus inoculated were kept at room temperature, and the other three were placed at 25°. Of the latter set one tube (1:1) showed a good, rich growth nine days later. Another of these tubes (3:1) on the same day showed some growth, but this was not as abundant as in the former probably on account of the large amount of water of condensation. Two tubes of the room set showed a good growth on October 8 (twenty days.)

Generation III.—This was made on September 28 from the rich 25° culture of the preceding generation. In this and subsequent inoculations the ordinary platinum loop was used. The blood agar employed was 1:1, and 2:1. Four tubes of each kind were inoculated. A set of two tubes, one of each kind, was set aside at room temperature. A like set was placed at 34°, and the remainder at 25°. Every one of these tubes gave a good growth. The 34° cultures developed rapidly, but died out shortly after the seventh day. One of the 25° cultures was alive on the twenty-fifth day, while one of the room cultures showed a few motile masses on the twenty-ninth day.

Generation IIIa.—This was inoculated on October 8 from one of the room cultures of Generation II. When examined on October 23, and again on October 27, many active single trypanosomes, as well as motile masses, were found. This material was injected on October 28 into four white mice (see Table I).

Generation IV.—This was made on October 2 from one of the 34° cultures mentioned above. The culture tube (2:1) was developed for nine days at 34° , and gave a very rich growth which persisted for thirteen days. A portion of this culture was injected on October 11, intraperitoneally, into a white mouse, with wholly negative result.

Generation IVa.—This was made on October 5 from one of the 25° cultures (1:1) of Generation III. Three tubes, 1:2, 1:1, and 2:1, were inoculated. These were developed at 25° for nine days, at which time the growth was abundant in the last two tubes.

A flask, 10 cm. in diameter, containing blood agar (2:1), was inoculated on October 6 and developed at 25° . It gave a very good growth. The contents of this flask were injected on October 14 into five white mice. The result of this injection will be referred to later. Another flask culture (IVb), inoculated on October 15 and kept at 25° , gave a good growth and was used on October 25 to inoculate flask culture Vb. On November 6 a portion of the fluid was injected into two white rats (Nos. 9 and 10).

This same flask culture (IVb) served also on October 29 for starting another flask culture (Vc).

A third flask of blood agar (3:2) was inoculated on October 15 from a 25° culture (1:1) of Generation III. On October 28 it showed a very fair growth.

Generation V.—This was made on October 11 from a 34° culture of Generation IV. The blood agar employed was 1:2 and 2:1, and the tubes after inoculation were kept at room temperature. Nine days later the first of these tubes showed many degenerations and only a few motile forms. The 2:1 blood agar showed no growth even on October 27. The failure to obtain a growth in this case may be due to the large amount of water of condensation present, or, what is more likely, the 34° culture from which the transplantation was made was in a too enfeebled condition, as indicated by its failure to infect a mouse.

Generation Va.—This was a flask culture (3:2) and was made on October 15 from the flask culture of October 6 mentioned under Generation IVa. The flask was kept at 25° for six days,

at which time it was very rich in trypanosomes. After removing a portion of the fluid for injection into a rat and a mouse (see Table II), the flask was placed at 34° for five days. On October 26 it showed no motile forms, and only masses of round bodies were present. The higher temperature in this case clearly destroyed the culture. This material was then injected into two white rats (Table III). Generation Vb was started on October 25 from one of the flask cultures of Generation IV. The medium (1:1) was contained in a flask, 10 cm. in diameter. For inoculation of animals with the culture see Table III.

Generation Vc was also made as a flask culture (1:1) on October 29 from Generation IVb.

Generation VI.—A flask containing blood agar (1:1) was inoculated on October 23 from Generation Va, developed six days at 25°, and two days at 34°, and was set aside at 25°. Six days later trypanosomes were found, though in small number. On November 10 the culture was very rich. The slowness with which the growth came on is due probably to the exposure at 34°.

Generation VIa.—A flask culture (3:2) was made on November 12 from Generation Vc. When left at 25° for four days it showed a fair growth; on the eleventh day it was very rich.

Generation VII.—A flask containing blood agar (1:1) was inoculated on November 10 from the preceding and placed at 25°. Actively growing trypanosomes were noted on November 16.

Generation VIIa.—A flask culture (3:2) was made November 16 from Generation VIa. In seven days at 25° it was quite rich in actively motile trypanosomes and was used to start Generation VIIa.

Generation VIII.—Three flask cultures were made on November 17 from Generation VII and set aside at 25° for six days, at which time a most excellent growth of trypanosomes was obtained. The flasks, however, developed mould contamination.

Generation VIIIa.—Two blood agar flasks were inoculated on November 23 from Generation VIIa. On December 4 both flasks showed a very rich growth and served to start Generation IX. On December 5 another set of blood flasks was inoculated giving Generation IXa.

CULTURE B.

Generation I.—The blood of a white mouse, rich in trypanosomes, was drawn from the heart immediately after death and transferred to two blood agar tubes (1:1), which were then set aside at room temperature (September 7). Again, as in the first generation of Culture A, the original trypanosomes gradually disappeared so that on the ninth day only one individual could be detected under a whole cover glass. On September 29 (twenty-second day) many active rosettes were found and persisted up to October 8 (thirty-one days). Only one of the two tubes inoculated gave a growth.

A second generation made on September 29 showed a fairly rich growth on October 27. A third generation made on October 29 showed no organisms on November 6.

CULTURE C.

This culture is of interest, as it was obtained from a white mouse which was probably dead for some hours, since there were only about two trypanosomes present in each field of the microscope. It shows that, given the proper soil, even a few trypanosomes can be induced to grow on the artificial medium. The inoculation was made on September 10 into two tubes of blood agar (2:1), which were then placed at room temperature. Here again the organisms originally planted disappeared, so that when examined four days later nothing could be found. On September 29, the nineteenth day, one tube showed several small rosettes; on October 8 the growth was quite rich, and a few motile trypanosomes were still present on October 20 (fortieth day).

A second generation was made on September 29 on blood agar (1:1) and developed at 25°. On October 11 it showed a fairly rich growth which, when injected into a mouse, caused death in eight days.

Generation IIa.—On October 8 four tubes of blood agar (1:3, 1:1, 2:1, and 3:1) were inoculated and kept at room temperature. On October 27 the first of these showed no growth, while the other three were well developed, the last perhaps being the best.

Generation III.—A flask containing blood agar (1:1) was inoculated on October 27 and set aside at 25°. When examined on November 4 it showed many actively motile small rosettes.

CULTURE D.

The special interest which attaches to this culture is the fact that it was developed on a very weak laked-blood agar. On ordinary blood agar we have not been able to grow *Tr. brucei* when the amount of blood was less than 2:3. The blood was laked by adding 1 volume of distilled water to 3 of blood and keeping the mixture for several hours at 37°. Equal volumes of the laked blood and Thalmann's agar were used for the inoculation.

The blood, very rich in trypanosomes was drawn from the heart of a white mouse just before death and transferred to thirteen tubes (September 12). Hesse's, Thalmann's, and ordinary agar were used. Some of these agar tubes were mixed with defibrinated blood, while others were mixed with the laked blood. Of this set only two tubes developed a growth. One of these was the Thalmann laked-blood agar mixture; the other was on an ordinary blood agar (1:1). The cultures were planted on September 12, and when examined on the sixth, and again on the seventeenth day were wholly negative. On October 8 the laked-blood agar showed several active rosettes and contained active forms even on October 27 (45 days). On October 11, when twenty-nine days old, a portion of the fluid was injected into mouse No. 4 (see Table I), but no infection took place. On the same day transplantations were made to three blood agar tubes, but none of these showed any growth on November 6. The culture had evidently become too weak to grow or to infect a mouse.

The foregoing details furnish a sufficient demonstration of the fact that the *Trypanosoma brucei* can be cultivated outside of the living body. The cultures are, however, by no means as readily obtained as in the case of the rat trypanosome. We can safely say that the latter organism can be cultivated every time from an infected animal. This we have repeatedly done during the past

six months without any failure. In every case the trypanosomes developed with certainty and in abundance.

The rat trypanosome finds the conditions suitable even when the amount of the blood in the medium is small. Thus it will grow on blood agar mixtures of 1:2, or 1:5, or even 1:10. The best conditions undoubtedly obtain with a 2:1 blood agar, on which the growth seems to be more rapid and more luxuriant.

The Nagana trypanosome, on the other hand, will only exceptionally grow on a medium which contains less than half its volume of blood. The best results seem to be given with 2:1 and even 3:1 blood agar mixtures. But even on these media the growth is by no means certain. Sometimes only one or two tubes develop out of a large number, although the medium is apparently the same in all of the tubes. This difficulty in causing the trypanosome to grow is best seen in the fact that we have succeeded in obtaining cultures from only four of the first fifty animals tested.

A noteworthy fact in connection with the starting of the initial cultures has already been alluded to. The original trypanosomes planted on the blood agar die out almost completely; so much so that an examination may fail to show any living forms, and thus give the impression that the culture is a failure and lead to the discarding of the tubes. And yet a few surviving organisms eventually succeed in adapting themselves to the changed condition, and from these the culture begins. The whole phenomenon is very suggestive of a survival of the fittest.

It has been shown that the cultures developed at the room temperature persist the longest. Several of these cultures contained living forms as late as the forty-fifth day. A temperature of 25° is well adapted for developing the cultures, but they do not last as long as those just mentioned. The trypanosomes may grow at 34°, but here again the cultures die out still earlier. A culture which has been grown at room temperature or at 25° when transferred to 34° dies out in a few days. It would seem from these facts as if an injurious substance persisted in the medium, and that its action was more marked the higher the temperature at which the growth took place. It is also possible that the waste products of the organism exert such an action.

The alteration which *Tr. brucei* undergoes in old cultures is brought out most clearly in their behavior to animals. It will presently be shown that, while the young cultures are almost as virulent as fresh Nagana blood, they become less and less virulent as they age, and, eventually, though living, may be wholly non-infectious.

INFECTION EXPERIMENTS.

It has already been pointed out that the virulence of blood from Nagana animals rapidly decreases, and that it disappears completely within three or four days after the removal of the blood from the animal. Living, motile trypanosomes may, however, be found in such material for five or six days. Our own experiments, made under different conditions, show: (1) that the original or surviving trypanosomes may retain their virulence for a greater length of time; (2) that virulent and non-virulent cultures may be obtained; and (3) that vaccination by means of non-virulent cultures is probable.

When trypanosome blood is transferred to blood agar tubes, the greater number of the trypanosomes which are thus carried over die out very rapidly, within two or three days, but some more hardy individuals survive for seven, ten, fourteen, or even eighteen days, without any apparent multiplication taking place. These survivals gradually lose their virulence, so that eventually no infection may follow the injection of motile trypanosomes. The similar behavior of blood, preserved in an ice-box for three days and showing slightly motile trypanosomes, has been noted by Laveran and Mesnil. In both instances the loss of virulence may be ascribed, with good reason, to the attenuation of the original surviving cells. On the other hand, it will be shown that pure living cultures may be obtained which are equally non-virulent.

Experiment 1. The liquid in a blood agar tube was inoculated with the blood of mouse No. 1, and was kept at room temperature. It showed a few motile trypanosomes on the eighteenth day (July 14) and was then injected into the peritoneal cavity of a white rat. No infection followed. On August 3, nineteen days later, the animal was inoculated with virulent Nagana blood. Infection resulted, the same as in a normal individual; and on the

fourth day, when the parasites were very numerous, the rat was bled for the purpose of another experiment.

Experiment 2. The liquid contents of a tube, inoculated from mouse No. 16 and kept at room temperature for fourteen days, was injected into the peritoneal cavity of a white mouse, and, although some motile trypanosomes were present in the fluid, no infection followed. On October 14, forty-eight days later, it was reinoculated with Culture A, Generation IVa. The trypanosomes appeared in the blood a day or two later than in the controls (Nos. 9 and 10, which see), and death followed in eight and one-half days; the two controls dying in seven and seven and one-half days respectively. This would apparently indicate that some slight protection was imparted by the previous inoculation. This matter will be referred to again under immunity.

Experiment 3. The blood from a white rat (No. 21), extremely rich in trypanosomes, was inoculated into blood agar tubes on August 24. The contents of one of these tubes showed living parasites on September 2 (ninth day). A white mouse injected with this material died in ten days. At the time the animal was found it had been dead evidently for some hours, since no trypanosomes could be detected in its blood. To make sure that it died of Nagana another mouse was inoculated with its blood, and died of the disease on the seventh day. Inasmuch as the number of living trypanosomes injected into the latter animal was probably less, since no living forms could be recognized, than was used for the first mouse, it follows that the long course of the disease, ten days, is not due to the injection of a few, but rather to markedly attenuated parasites.

Experiment 4. On September 9 the fluid contents of three tubes, kept at room temperature for two, four, and eight days respectively, were injected into three white mice. The tubes had been inoculated with blood rich in trypanosomes. Mice Nos. 1 and 2, which received the two- and four-day-old fluids respectively, died on the fifth day of Nagana. Mouse No. 3, which received the eight-day fluid, containing living trypanosomes, developed no infection. On September 27 it was reinoculated, this time with virulent Nagana blood, and died in four days.

Experiment 5. On October 17 six tubes of blood agar were inoculated with the blood of a rat, drawn during life and rich in parasites. By inoculating a set of tubes with the blood of one animal and testing these at definite intervals, it seemed as if better results could be obtained than with tubes inoculated at different times with blood from different animals as in the preceding experiment. The tubes were placed at 25°. On the third, fifth, eighth, and tenth day the contents from one of these tubes was injected into a white rat. The rat which received the fluid from the tube three days old showed trypanosomes in its blood on the third and died on the seventh day. The rat which received the contents of the five-day tube showed a very few trypanosomes in its blood on the eighth and died on the twelfth day. Motile trypanosomes were present in the tubes on the eighth and also on the tenth day, but the rat inoculated with fluid from the eight-day tube showed no infection, whereas the one which received the ten-day fluid died in seven days. Its spleen was small, and no trypanosomes could be detected in its blood. Nevertheless, a rat inoculated with this blood died in six days, and trypanosomes were found in its blood. Evidently, survivals ten days old may infect. The failure of the eight-day fluid to infect may have been due to a greater resistance of the rat or to some slight variation in the medium which thereby weakened the parasite, more so than did the ten-day fluid.

The above experiments show that the virulence of surviving trypanosomes, in blood agar tubes, may be maintained for a longer period than under ordinary conditions, since infection was obtained on the fifth, and even on the ninth and tenth day. Under the conditions which obtain in the tubes the organisms, however, usually become non-virulent after the fourth or fifth day. The long duration of the disease (ten and twelve days), and the further fact that the older cultures, though containing living trypanosomes, are devoid of virulence, show that a progressive attenuation is taking place.

It is a well-known fact that a blood may be highly virulent without containing any trypanosomes, recognizable as such under the microscope. A single virulent cell, which perhaps may have

escaped observation, is therefore able to produce death in a very short time. An entirely different result follows the injection of even large numbers of living trypanosomes which are either, as in the above experiments, survivals, or actual cultures weakened by prolonged exposure to unfavorable conditions. The period of incubation and the duration of the disease is prolonged, or else no infection results. It is evident that these facts are explainable only on the supposition that actual attenuation takes place. We have not as yet been able to obtain a culture which could be said to be attenuated throughout its development in the tube. The cultures, as will be shown presently, are virulent in the early stages of their growth, after which they weaken and even become non-infectious.

Laveran and Mesnil have shown that virulent Nagana blood, when diluted with 50,000 parts of physiological salt solution and injected into the peritoneal cavity of a mouse, causes death in six and one-quarter days. They have also shown that when blood which is very poor in parasites is injected into mice and rats the trypanosomes may not appear in their blood before the fifth to the seventh day. The injection, into mice, of blood which has been kept at room temperature for thirty-six to forty-eight hours is followed by the appearance of the parasites in the blood on about the ninth day. They have noted the same delay in the appearance of the parasites in the blood after the injection of material which had been kept in an ice-box, or which had been heated to 40-43°, or exposed to the action of injurious chemicals. The prolonged period of incubation in these instances is, in view of our own results, due to a rapid attenuation of the trypanosomes. The same explanation may hold true in the case of Bruce's experiments on dogs, in which a prolonged duration of the disease followed the bites of infected flies which had been kept for twelve to forty-eight hours.

INOCULATION OF PURE CULTURES.

In the previous set of experiments the inoculations were made with the surviving trypanosomes as found on blood agar media where apparently no multiplication had taken place. When, how-

ever, undoubted cultures were at last obtained, it became necessary to test these on animals; first, with the object of demonstrating that the cultures were really those of *Trypanosoma brucei*; and, second, to confirm the conclusion arrived at from the preceding experiments with reference to the existence of attenuated or non-virulent forms.

It was soon found that the virulence of the trypanosomes was not a fixed quality but that it is easily influenced by altering the external conditions, such as temperature, the duration of the cultivation and possibly the composition of the medium. These facts had already been foreshadowed in the results obtained by inoculation of the survivals.

The inoculation of mice and rats with the pure cultures completed the evidence necessary to show that the organism cultivated was in reality *Trypanosoma brucei*, and, at the same time, supplied the final proof, if any was needed, that this trypanosome was the cause of Nagana. This organism has now been cultivated *in vitro* for one hundred days, through eight generations, and, as will be seen from Table II, possesses a virulence not much inferior to that of trypanosome blood. There can be no doubt that, given the best possible conditions, it can be cultivated in fully as virulent a form as that met with in living blood.

The intraperitoneal injection of virulent cultures causes death in mice and rats usually in seven or eight days, exceptionally in as late as eleven days. When it is remembered that these animals usually die in from three to five days after the injection of virulent blood, it will be seen that the cultures employed were already somewhat weakened. The retarded death is not due to the injection of a small number of trypanosomes, since in all these experiments the cultures may be characterized as extremely rich in actively motile parasites.

The virulence of one culture was such that a rat died in three and one-half days (see Table III). Two mice (Nos. 8 and 4a) inoculated with another culture died in three and one-half and five and one-half days respectively. Mouse No. 8 had received seventeen days before an injection of a non-virulent culture, while mouse No. 4a served as a control for the last injection. This same

virulent culture was injected at the same time into another control mouse (4b), and an examination of its blood, three days later, resulted in finding only one trypanosome in the cover-glass preparation examined. Subsequent examinations, made at intervals of two days, failed to show any trypanosomes. This is the only time that we have found the parasite in the blood without fatal infection following. It may be that the single cell found was a survival of the material injected, and was hence incapable of multiplication. In that event this mouse must be credited with possessing a most unusual degree of resistance.

It should be stated, however, that in this experiment four mice were inoculated at the same time and with the same material. Two of these had survived previous inoculations of non-virulent cultures, and the other two served as controls. One vaccinated mouse and one control died; the others remained alive. It would seem as if an interchange took place, and that the two deaths were those of the controls, but this could hardly have occurred, since special care was exercised to prevent such accidents.

In mice and rats, after the injection of trypanosome cultures, the parasites appear in the blood, usually on the fifth day, exceptionally as early as the third and as late as the seventh and even the ninth day. After the trypanosomes once appear in the blood they rapidly multiply, with the single exception noted above, and become exceedingly numerous just before death. In from two to four days after the trypanosomes appeared in the blood the animals died.

The trypanosomes which develop in the blood of an animal after an injection of a pure culture are identical, in every respect, with those met with in Nagana. The highly refracting globules, usually two or three in number, which are seen in the cultivated trypanosome are not met with in the inoculated animals. We are inclined to consider these refracting globules as evidence of unfavorable environment, and hence as an indication of partial attenuation.

The isolation of the trypanosomes from the blood of animals infected with the pure cultures is as difficult a task as in the case of those inoculated directly with Nagana material.

The cultures employed for the following inoculations were rich in actively motile trypanosomes. The injections were made, as usual, into the peritoneal cavity. It will be seen that only the thirteen- and twenty-day cultures produced infection. It would seem as if a room culture, after two or three weeks, gradually became deprived of its virulence. In the case of the first generation, it should be remembered that no appreciable growth takes place until after eighteen or twenty days. The unfavorable conditions with which the trypanosomes are struggling may be looked upon as being responsible for the absence of virulence. The subsequent generations, having accommodated themselves to these conditions, regain, for a time at least, their pathogenic properties.

TABLE I.

Showing Results of Inoculation of Cultures of *Trypanosoma Brucei* Developed at Room Temperature.

	CULTURE AND GEN- ERATION	BLOOD AGAR	AGE OF CULTURE	DATE OF			TIME OF DEATH
				Inocu- lation	Appear- ance of Trypano- somes	Death	
Mouse 1	AI	2:3	34 days	Sept. 30	0	0	Recovered
Mouse 4	DI	1:1 ⁵	29	Oct. 11	0	0	Recovered
Mouse 5	A II	2:1	23	Oct. 11	0	0	Recovered
Mouse 7	A III	1:1	13	Oct. 11	Oct. 16	Oct. 19	8 days
2d inject'n mouse 8	A IIIa	3:1	20	Oct. 28	Oct. 31	Nov. 1	3½ days
2d inject'n mouse 4	A IIIa	3:1	20	Oct. 28	0	0	Recovered
Control mouse 4a ..	A IIIa	3:1	20	Oct. 28	Oct. 31	Nov. 3	5½ days
Control mouse 4b ..	A IIIa	3:1	20	Oct. 28	Oct. 31 ⁶	0	Recovered

The cultures employed in the following inoculations were developed in an incubator room at 25°, and were extremely rich. It will be seen that all of these cultures proved fatal with the exception of one. The mouse used for this inoculation had survived a previous inoculation of a room culture (see Table I). It was given a second injection of an eighteen-day culture, presumably not very virulent, in order to test its immunity. Inasmuch as all the fluid in the culture tube was used for this injection, it was not possible to make a control. The survival of the animal may be due, therefore, either to a condition of immunity or to the non-virulence of the culture.

⁵ The medium in this case was laked-blood agar. The blood was partially laked by the addition of sterile distilled water (1:3), the mixture being kept at 37° for several hours.

⁶ Only one trypanosome found in preparation on the third day; none after that date.

TABLE II.

Showing Results of Inoculation of Cultures of *Trypanosoma Brucei*
Developed at 25°.

	CULTURE AND GEN- ERATION	BLOOD AGAR	AGE OF CULTURE	DATE OF			TIME OF DEATH
				Inocu- lation	Appear- ance of Trypano- somes	Death	
Mouse 3	AIII	2:1	7 days	Oct. 5	Oct. 14	Oct. 16	11 days
Rat 1	AIII	2:1	7	Oct. 5	Oct. 16	11
Control mouse 9....	AIVa	3:2	9	Oct. 14	Oct. 19	Oct. 22	7½
Control mouse 10....	AIVa	3:2	9	Oct. 14	Oct. 19	Oct. 21	7
2d inject'n mouse 1	AIVa	3:2	9	Oct. 14	Oct. 21	Oct. 23	9
2d inject'n mouse 2	AIVa	3:2	9	Oct. 14	Oct. 19	Oct. 24	9½
2d inject'n mouse 16	AIVa	3:2	9	Oct. 14	Oct. 21	Oct. 23	8½
Mouse 11	A Va flask	3:2	6	Oct. 21	Oct. 26	Oct. 29	7½
Rat 2	A Va flask	3:2	6	Oct. 21	Oct. 28	Nov. 2	11½
Mouse 6	CII	1:1	12	Oct. 11	Oct. 16	Oct. 19	8
2d inject'n mouse 5	AIV	1:1	18	Oct. 23	0	0	Recovered
Rat 5	A Vb flask	1:1	10	Nov. 4	Nov. 8, A.M.	3½ days ⁷
Rat 9	AIVb flask	3:2	22	Nov. 6	Nov. 9	3 days ⁸
Rat 10	AIVb flask	3:2	22	Nov. 6	Nov. 13	Nov. 16	10
Control mouse 16....	AV flask	1:1	18	Nov. 12	Nov. 18	Nov. 21	9
Control mouse 17....	AV flask	1:1	18	Nov. 12	Nov. 20	Nov. 22	10
3d inject'n mouse 4	AV flask	1:1	18	Nov. 12	Nov. 18	Nov. 21	9
2d inject'n mouse 4b	AV flask	1:1	18	Nov. 12	Nov. 18	Nov. 20	8

It is very probable that the cultures when kept for three or four weeks at 25° will lose their virulence, but on this point we have as yet no definite data.

TABLE III.

Showing Results of Inoculation of Cultures of *Trypanosoma Brucei*
Developed or Exposed at 34°.

	CULTURE AND GEN- ERATION	BLOOD AGAR	AGE OF CULTURE	DATE OF			TIME OF DEATH
				Inocu- lation	Appear- ance of Trypano- somes	Death	
Mouse 2	AIII	2:1	1 d. 25°; 3 d. 34°	Oct. 2	0	0	Recov.
Mouse 8	AIV	2:1	9 d. 34°	Oct. 11	0	0	Recov.
Rat 3	A Va flask	3:2	6 d. 25°; 5 d. 34°	Oct. 26	0	Nov. 9	14 d. ⁹
Rat 4	A Va flask	3:2	6 d. 25°; 5 d. 34°	Oct. 26	0	Nov. 9 A.M.	13½ d. ⁹
Control rat 5	A Vb flask	1:1	10 d. 25°	Nov. 4	Nov. 8 A.M.	3½ d.
Rat 6	A Vb flask	1:1	10 d. 25°; 1 d. 34°	Nov. 5	Nov. 11	Nov. 14	9 d.
Rat 7	A Vb flask	1:1	10 d. 25°; 2 d. 34°	Nov. 6	0	0	Recov.
3d injection rat 8.	A Vb flask	1:1	10 d. 25°; 3 d. 34°	Nov. 7	0	0	Recov.
Mouse 5	A Vb flask	1:1	10 d. 25°; 3 d. 34°	Nov. 7	0	0	Recov.
Mouse 12	A Vb flask	1:1	10 d. 25°; 4 d. 34°	Nov. 8	0	0	Recov.
Mouse 13	A Vb flask	1:1	10 d. 25°; 5 d. 34°	Nov. 9	0	0	Recov.
Mouse 14	A Vb flask	1:1	10 d. 25°; 6 d. 34°	Nov. 10	0	0	Recov.
Mouse 15	A Vb flask	1:1	10 d. 25°; 6 d. 34°	Nov. 10	0	0	Recov.

⁷ This served as a control for set of inoculations made with the same material heated at 34°. See Table III.

⁸ No trypanosomes could be found in the blood. Moreover, the spleen was small. To check this result the blood of this animal was injected into a mouse. No infection followed, and for that reason the death of Rat 9 cannot be ascribed to Nagana.

⁹ See below.

Notwithstanding that the cultures develop more rapidly at 34° than at lower temperature, it is evident from the above table that they are non-virulent. The cultures used for Mice 2 and 8 were actively motile. Such cultures developed from the start at 34° rapidly reach their maximum growth, after which they die out.

The culture used for Rats 3 and 4 was first developed at 25° , after which it was placed at 34° . When examined at the end of five days, only round bodies were present, and there was an entire absence of motile forms. These animals therefore really received injections of dead cultures. Although their blood was repeatedly examined, no trypanosomes could be detected. Both rats died on the fourteenth day. The spleen was small, and no trypanosomes could be found in the blood. A mouse was inoculated with the blood of one of these rats and developed no infection.

The death of these animals, unless it be a mere accident, can be explained only on the supposition that the large amount of dead trypanosomes injected caused a fatal intoxication.

In order to test the action of a temperature of 34° upon cultures first fully developed at a lower temperature, a flask culture grown at 25° for ten days was selected, and after injecting a rat (No. 5) with a portion of the fluid, for the purpose of a control, it was set aside at 34° . On each of the following days a portion of the fluid was removed and injected into rats. The result of this experiment is shown in Table III. The flask, after being kept at 34° for three days, began to show involution forms. Moreover, the fat-like globules within the trypanosomes increased in number and size. Motile trypanosomes were still present, though few in number, on the fifth day. No motile forms and only degenerations were present on the sixth day. Two drops of the fluid were injected into a mouse (No. 14), and the remainder of the fluid about $\frac{3}{4}$ c.c., was injected into another mouse (No. 15).

It will be seen from the table that the control rat, No. 5, died in three and one-half days after inoculation. Its spleen was somewhat enlarged. The rat had been dead for some hours, and an injection of a suspension of the internal organs failed to infect a mouse. The trypanosomes had clearly disappeared from the

cadaver. The same material, though very rich in motile trypanosomes, after an exposure of twenty-four hours at 34° gave a retarded infection, seen in the fact that the parasites did not appear in the blood until on the sixth day, and death did not occur until the ninth day. The other animals of the series showed no infection.

This experiment shows that an exposure of less than forty-eight hours at 34° is sufficient to destroy the virulence of the cultivated trypanosome, which, however, may remain alive and very motile at this temperature for about five days.

The results of another experiment go to show that, while the 25° culture is attenuated by an exposure of two days at 34° with reference to the new rat, it is still sufficiently virulent to kill rats whose resistance had been lowered by previous inoculation.

Inasmuch as the trypanosomes grow normally in the bodies of animals at a temperature of 37 to 39° , and, under such conditions, maintain their virulence indefinitely, it follows that the temperature is not the only factor which weakens the trypanosome cultures. It has been shown that in whole blood, or even in blood diluted with salt, or citrate solution, or with serum, the trypanosomes die out in anywhere from a few hours to five or six days. The death of the trypanosomes is not due to their inability to multiply outside of the body, but rather to the presence, in more or less concentrated form, of injurious substances probably formed as a result of the death changes in the composition of the blood. In the blood agar medium the injurious action of these products may be considered to be largely neutralized by the agar constituents. That this neutralizing or antitoxic action is not complete is seen in the fact that the room cultures lose their virulence as they age. This effect is more accentuated and is hastened in cultures developed at a higher temperature. As yet we know nothing about the nature of these toxic or antitoxic substances. We are, however, of the opinion that toxic salts or ions are responsible for the rapid destruction of the trypanosomes in dead blood, and, if such be the case, it will be possible to obviate their action. Experiments along this line are now being conducted.

IMMUNITY.

The existence of living and non-virulent cultures of *Trypanosoma brucei* indicates the possibility of immunizing animals by means of such cultures. Thus far no means of protection has been found against the ravages of Nagana, nor for the equally important diseases Surra and Caderas. Even experimentally it has not been possible to protect susceptible animals against infection. Sheep, goats, and cattle are less susceptible, and recovery from the disease protects against subsequent inoculation.

Koch suggested the possibility of attenuating the Nagana trypanosomes by repeated passage through different species of animals, and Schilling endeavored to apply this method, but his results may be said to be highly unsatisfactory, and it is very doubtful if any real protection is obtainable by such procedure.

We have already shown that the Nagana trypanosome can be attenuated by cultivation *in vitro*, and especially by exposure of such cultures to a temperature of 34°. Whether such cultures are able to impart to animals a sufficient degree of immunity so as to protect them against inoculation with virulent blood, we are as yet unable to state. Our own experiments along this line are as yet too few to justify any positive statement. It is our intention to follow out this line of inquiry, and such results as may be obtained will form the subject of a special article.

The result of one experiment of this kind has already been alluded to and is summarized in Table I. It will be seen that the control and treated animals behaved alike; that is to say, one of each died and one of each survived. The control which survived showed a single trypanosome in a cover-glass preparation on the third day, but none thereafter. The treated mouse, No. 4, which recovered, at no time showed the parasites in its blood. The contradictory results of this experiment suggest that an interchange of the animals had in some way taken place, and that the surviving mice were those which had been treated. In view of the great care which was taken to prevent such interchange, it hardly seems probable that this could have occurred. It should perhaps be stated that each of the animals received $\frac{1}{4}$ c.c. of a very

rich culture, and that all four injections were made from one charge of the syringe. Other experiments now under way will help to give a definite solution in this problem.

Another immunity experiment is given in Table II. Two control mice, Nos. 9 and 10, and three treated mice, Nos. 1, 2, and 16, were injected with the same culture. It will be noted that the controls died in seven and seven and one-half days, while the treated mice died in eight and one-half, nine, and nine and one-half days. The single treatment to which these mice were subjected apparently gave some slight protection. The failure to secure perfect immunity may be ascribed to the fact that the material injected in the first inoculation was too feeble. Thus mouse No. 1 (see Table I) received a first generation, thirty-four days old. Mouse No. 2 (Table III) received an obviously feeble culture, since it was developed only for one day at 25°, and then for three days at 34°. Mouse No. 16 received on August 27 an injection of the fluid of a tube, inoculated fourteen days before with the blood of a Nagana rat, and containing only surviving trypanosomes.

It is quite likely that mouse No. 5, as shown in Table II, had acquired considerable protection against a very rich and probably virulent culture as the result of a previous injection of a twenty-three-day room culture (Table I). Repeated injections of attenuated cultures will undoubtedly be necessary in order to secure immunity to virulent cultures and to Nagana blood.

MORPHOLOGY.

The appearance of *Trypanosoma brucei*, in cultures, is extremely characteristic and is wholly unlike that of *Tr. lewisi*. When the culture fluid containing the latter organism is examined under a cover-glass, the individual cells will be found to be clear and almost homogeneous. *Trypanosoma brucei*, on the other hand, invariably shows two large, highly refracting globules at the anterior or flagellated end. Occasionally a third and smaller granule is found toward the posterior end. The globules will be found to increase in number and in size when a fully developed

culture, grown at room temperature, or at 25° , is placed at 34° for several days. In such a case the globules may measure 0.8 and even 1μ in diameter. A single cell may contain five or six of the large bodies, besides many very small granules. These globules are slightly greenish in color, and when preparations are treated with osmic acid, for twenty-four to forty-eight hours, they darken perceptibly, though at no time do they become perfectly black. It would seem as if these bodies consisted largely of fat, though the presence of paramylum, as in other flagellates, is possible.

The presence of these globules in cultures and their entire absence in the trypanosomes found in the animal body would seem to indicate an alteration in the functional activity of the cells. The fact that the globules are increased in number and in size when the culture is exposed to 34° , in view of the fact that such exposure rapidly destroyed the virulence of the organisms, certainly goes to support this conclusion.

It is probable that, with an improvement in the culture medium, trypanosomes will be obtained free from globules, in which case they will correspond to or resemble exactly those found in the animal. As yet, however, we have not been able to obtain a single culture which did not contain these bodies.

The *Tr. lewisi* as found in cultures varies greatly in size. It is not unusual to find minute forms which, not counting the whip, are but 1 or 2μ in length. There are others which are typical in form which are not much longer than the diameter of a red blood cell. While most of the spindle-shaped cells range from 15 to 20μ in length, some trypanosomes can be found, at times, which are 50 to 60μ long. The existence of the small form accounts for the fact that we have repeatedly been able to infect rats with Berkefeld filtrates of such cultures.

Trypanosoma brucei, on the other hand, shows much less variation in size. The living cell usually measures about 15 to 17μ in length. The *Tr. brucei* is longer and proportionately narrower than the *Tr. lewisi*. The flagellum in the living cell is by no means as distinct and as long as that of the latter organism.

The motion of the two trypanosomes is entirely different. Free swimming or "swarming" forms are common in the case of *Tr. lewisi*. By means of the long flagellum this organism is capable of moving about with great rapidity and in an almost straight line. The motion of *Tr. brucei* is slow and wriggling, and only exceptionally is a slowly progressive form observed. The wave motion slowly passes along the thick, undulating membrane, and gives the appearance of a spiral rotation to the entire cell.

The most striking difference is seen in the appearance of the colonies or aggregates. In the case of *Tr. lewisi* the individual cells are usually grouped in rosette-like aggregations or colonies. These colonies may consist of a dozen or so, or even of hundreds of cells. Again, a number of these colonies may partially coalesce and give rise to enormous multiple colonies which may consist of many thousands of individuals. The colonies present an orderly, symmetrical, and beautiful appearance. The individual cells composing a rosette are spindle-shaped and have a more or less active swaying motion. The flagella in such a colony are not recognizable on the periphery, as in the case of agglutination rosettes. Such rosettes stained by Romanowsky's method show the centrosome close to and below the nucleus; that is to say, the centrosome is nearer the center of the rosette than is the nucleus. This would seem to indicate that the peripheral end is the anterior end, and that the flagella develop there when the cultural conditions are most nearly perfect. This would seem to be the case in the small multiplication rosettes observed in the living blood of the rat.

On the other hand, the cultures of *Tr. brucei* will usually show many cells, in pairs, united by their posterior ends and corresponding to the so-called conjugation forms of Bradford and Plimmer. Larger masses or colonies consisting of ten to twenty cells are met with. The individuals composing these colonies have not the rigid spindle form of *Tr. lewisi*, but are long, narrow, and writhing, and the effect of the whole mass is very suggestive of the snakes on a Medusa head. The flagella appear to be directed outward.

The mode of multiplication of *Tr. brucei* and of *Tr. lewisi* need not be considered at present, inasmuch as this matter will be treated in a subsequent paper.

SUMMARY.

The results of this investigation may be briefly summarized as follows:

1. *Trypanosoma brucei* is more sensitive and more exacting in its requirements than is *Tr. lewisi*. It may, however, be cultivated, *in vitro*, in tubes, and in flasks, by applying the same method which was used for obtaining pure cultures of the rat trypanosome.

2. The chief difficulty consists in obtaining the first or initial culture. Of the first fifty animals from which cultures were attempted only four gave positive results. One of these cultures has been carried on continuously from August 27 up to December 5 (one hundred days), and in this time has been passed through eight generations. There is no reason why the culture should not be maintained indefinitely.

3. It is possible to separate *Tr. lewisi* and *Tr. brucei* when these are present simultaneously in the blood of an animal, by artificial cultivation, since the former will grow on a medium containing one-half or less of its volume of blood, whereas *Tr. brucei* will not grow under such conditions.

4. The cultural characteristics are such as to enable perfect differentiation between the two trypanosomes.

5. The actively growing cultures of *Tr. brucei* possess a virulence which is very nearly the same as that of the original Nagana blood. As the cultures age, and especially by exposure to a temperature of 34°, they become less virulent and eventually, though living, may fail to infect animals.

6. Repeated injections of the attenuated cultures will probably impart solid immunity. In this way it may be possible to secure protection against the ravages of Nagana.

7. The pathogenic action of the pure cultures of *Tr. brucei* demonstrates the causal relationship which this organism bears to Nagana.

8. It may be considered as certain that the method which has proved successful for *Tr. lewisi* and *Tr. brucei* will prove equally useful for other trypanosomes. It will be feasible to subject the trypanosomatic diseases to experimental studies which heretofore have been impossible. The results of these studies may lead to a clearer insight into the causation of protozoal diseases. It seems as if the time is not far distant when most of the pathogenic protozoa will be cultivated and studied in our laboratories in the same way as is now done with bacteria.

STUDIES IN PYROPLASMOSIS HOMINIS.*

("SPOTTED FEVER" OR "TICK FEVER" OF THE ROCKY MOUNTAINS.)

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INTRODUCTORY.

THE disease known in Montana as "spotted fever"¹ had been recognized as a distinct clinical entity by the physicians of the Bitter Root Valley for fifteen or twenty years, but the only article in a medical journal concerning it, published prior to 1902, was that by Dr. E. E. Maxey in the Portland *Medical Sentinel* for October, 1899. Papers, by Drs. R. Gwinn and G. T. McCullough, were read at the May, 1902, meeting of the Montana State Medical Association, but not published. These three described the clinical symptoms, but the pathology and etiology were not discussed.

May 12, 1902, the writers were engaged by Dr. A. F. Longeway, secretary of the Montana State Board of Health, to investigate the nature, causation, and means of prevention of the disease. Dr. Wilson arrived in Missoula on May 16, 1902, and Dr. Chowning on May 26, 1902. Both the writers remained in Missoula or

* Received for publication, Oct. 15, 1903.

¹ The name "spotted fever" as applied to the disease under consideration is an unfortunate one, since it has been applied to several other diseases of man. The name "tick fever," proposed by local newspapers when the hypothesis of transmission by ticks was advanced by the writers in 1902, seems to be fairly distinctive. It is open to the objection that it has been previously used as a synonym for "Texas fever" in cattle, and that it does not accurately indicate either symptoms or etiology. Since the disease appears to be the first described infection of man by a pyroplasma, the writers propose for it the name *pyroplasmosis hominis*.

the vicinity until July 14, 1902. During this time the writers studied seven cases clinically and performed six autopsies.²

During the last week of June, 1902, Dr. F. F. Westbrook, professor of pathology and bacteriology in the University of Minnesota, and director of the Minnesota State Board of Health Laboratory, and Dr. J. O. Cobb, surgeon of the United States Public Health and Marine Hospital Service, visited Missoula, and in company with the writers examined clinically case No. 96. They also examined fresh blood preparations from this case, and gross and microscopic specimens from previous autopsies and spermophiles.³

During the spring of 1903 the writers continued the investigation for the Montana State Board of Health, Dr. Wilson being in Missoula from April 22 to May 20, and Dr. Chowning from May 19 to June 19, 1903. During this period the writers studied clinically ten cases and performed two autopsies. They also collected data concerning two additional cases which were seen by the family physician only.

Dr. John F. Anderson, surgeon and assistant director of the United States Public Health and Marine Hospital Laboratory, studied with the writers five cases—including one autopsy—which occurred from May 1 to May 30, 1903. The writers also gave Dr. Anderson copies of their observations on cases Nos. 115-19 inclusive, two photographs of case No. 94, one photograph of case No. 117, and a map of the locality showing the location of cases.⁴

The writers desire to express their most sincere thanks to Dr. F. F. Westbrook, professor of pathology and bacteriology in the University of Minnesota and director of the Bacteriological Laboratory of the Minnesota State Board of Health, and to Dr. H. M. Bracken, secretary of the Minnesota State Board of Health

²A preliminary note on these investigations was published in the *Journal of the American Medical Association*, July 19, 1902, and a full account in the *First Report of the Montana State Board of Health*, December 31, 1902.

³WESBROOK, *Biennial Report Minnesota State Board of Health*, 1901-2; COBB, *U. S. Public Health and Marine Hospital Service Reports*, 1902, 1868.

⁴ANDERSON, "Preliminary Report," *American Medicine*, September 26, 1903; full report in forthcoming *Bulletin No. 15*, Laboratory of U. S. Public Health and Marine Hospital Service.



SHOWING

Position of Western Alaska

MAP

AREA INFECTED WITH SPOTTE



and professor of materia medica and therapeutics in the University of Minnesota.

The most generous assistance was also received from the State Universities of Minnesota and Montana, the State Boards of Health of Montana and Minnesota, the Northern Pacific Beneficial Association through its chief and assistant surgeons, Drs. J. J. Buckley and E. W. Spottswood, and from the physicians and citizens of Missoula and the Bitter Root Valley.

HISTORY OF THE DISEASE.

So far as can be determined, the first case of "spotted fever," "black fever," or "blue disease" in the Bitter Root Valley in Montana occurred in 1873. At this time there were but few white men in the valley. No authentic information of its occurrence among the Indians—who until 1890 inhabited the valley—has been obtained, though many old residents, including Indians, white trappers, traders, and Catholic priests, were consulted.

Since its first appearance probably 200 cases of the severe type have occurred, 70 to 80 per cent. of which have been fatal. During the spring of 1902, 18 such cases developed, 15 of which died and 3 recovered. During 1903 12 cases developed, 6 of which died and 5 recovered completely, while 1 died of acute gastritis after convalescence had been established for some time. The writers collected from the attending physicians data concerning 114 of these cases, and published them with a locating map in the *Montana State Board of Health Report*, 1902. The map is herewith reproduced, with the numbers of the cases occurring during 1903 added thereto. (See map opposite p. 32.)

LOCATION.

The cases of "spotted fever" in Montana are confined to the eastern foothills of the Bitter Root Mountains (a range along the top of which runs the Montana-Idaho state line); namely, on the western side of the Bitter Root Valley in an area from four to ten miles wide and fifty miles long. On the map seven cases are noted as occurring on the east side of the Bitter Root River. There is reason to believe, however, that each of these cases was

originally infected on the west side of the river. There is nothing to indicate where case No. 104 received its infection. Few cases have been known to originate in Montana outside of this territory, except eight in an area about a half-mile wide and ten miles long in the narrow cañon of Rock Creek about twenty miles east of the Bitter Root Valley. Two cases have occurred in a valley near Bridger, Mont., about two hundred and fifty miles east and seventy-five miles south of the Bitter Root Valley. Dr. Maxey's paper⁵ describes cases in Idaho mostly along the southern foothills of the Boise Mountains. The cases seem to be limited largely to the north side of the Snake River Valley from Seven Devils to Haley, and to occur from the latter part of March to the middle of July. After the publication of the writers' preliminary report, W. M. Wood, major surgeon in the United States army, presented the writers with a copy of a series of letters which he received from a number of physicians in Idaho in 1898 in response to his inquiries concerning "spotted fever" in that state. Major Wood presented a symposium of these letters in a report to the surgeon-general of the United States army in 1898, but, so far as the writers are aware, this report has not been published. This symposium, with Dr. Maxey's paper and a number of communications received by the writers from physicians in Idaho, seems to make it certain that the disease known as "spotted fever" in Montana has also a definite distribution in Idaho, and that it resembles very closely the disease in Montana in all respects except in its comparatively very low mortality.

A personal letter from Dr. W. R. Kendall, major and surgeon in the United States army, San Francisco, describes cases seen by him in 1887, in the Quinn River Valley in Nevada, and a similar letter from Dr. J. J. Bradbury, Cody, Wyo., describes cases occurring near Cody and near Meeteetse, Wyo., during the spring of 1903. In both of these localities the disease is of the mild type.

The Bitter Root range of mountains is very rugged, the top being covered with snow until about July 1. The range on the east side of the valley is less rugged, though the snow remains

⁵ See p. 31.

almost as long in spring as on the west side. On the foothills the snow melts from sunny exposures as early as February, the bulk of it disappearing in April and May. The climate of the valley is very mild, as is evidenced by the many orchards of apple, pear, cherry, and plum trees. The altitude of the valley is about 3,500 feet above sea level. The population is made up largely of fairly well-to-do ranchers, the majority of whom have come from Missouri, Georgia, and the Carolinas. They are, as a rule, cleanly and healthy. The lumber industry is an important one, and many cases of "spotted fever" have arisen about saw-mills and on ground recently cleared of timber.

SEASON.

The disease occurs chiefly in the spring. The earliest recorded case began March 17, and the latest about July 20, though most cases occur between May 15 and June 15. There are no records of any cases occurring between July 20 and March 17, though there are rumors of some cases having occurred as early as February. The following table shows the distribution by months of the reported cases:

TABLE I.

Showing Distribution of Cases by Months.

March	-	-	-	-	6 cases	
April	-	-	-	-	24	"
May	-	-	-	-	46	"
June	-	-	-	-	35	"
July	-	-	-	-	5	"
Spring	-	-	-	-	10	" (but exact month uncertain)
Total	-	-	-	-	126 cases	

PREVIOUS CONDITION OF PATIENT.

The general health of the patient appears to have little part in determining susceptibility to the disease. A large number of cases give a history of recent exposure to wet or cold, or of over-exertion shortly before the attack, but in several cases all such history has been absolutely excluded. Many of the patients had suffered somewhat from indigestion and constipation immediately prior to the attack. In others no such condition existed.

SEX AND AGE.

The relationship of sex and age to susceptibility and mortality is shown in the following table:

TABLE II.

Showing Fatal and Recovered Cases Arranged with Reference to Age and Sex of Patients.

	MALES			FEMALES			Total Cases, Both Sexes
	Died	Recov- ered	Total	Died	Recov- ered	Total	
Under five years	4	4	8	5	..	5	13
Five to ten years	5	1	6	4	3	7	13
Ten to twenty years	5	3	8	5	6	11	19
Twenty to thirty years	13	4	17	3	4	7	24
Thirty to forty years	19	5	24	7	4	11	35
Forty to fifty years	6	2	8	1	1	2	10
Fifty to sixty years	2	1	3	1	1	2	5
Sixty to eighty years	4	..	4	2	..	2	6
Age not stated	1	..	1	1
Total	59	20	79	28	19	47	126

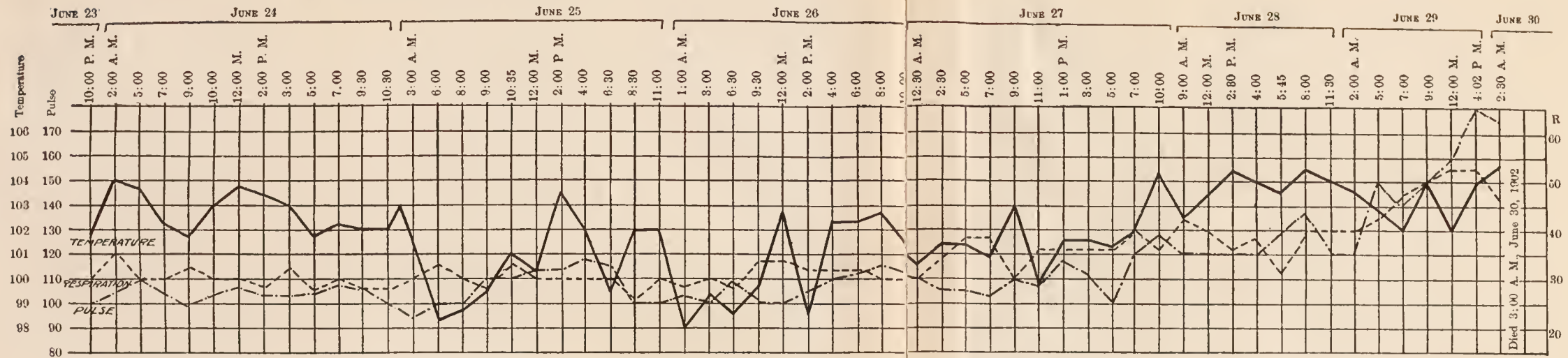
The cases are too few to warrant the drawing of elaborate conclusions where such varying factors are concerned. It is probable, however, that the large number of cases occurring in males of twenty to forty years and in females of somewhat younger age is due to the increased exposure to infection through their occupation or pleasure taking them outdoors in the foothills and mountains in the spring of the year.

TYPES OF THE DISEASE.

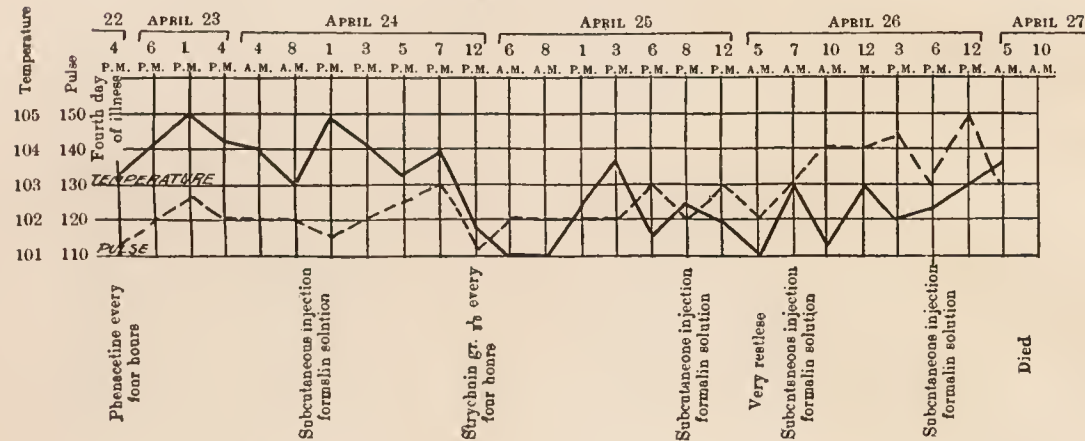
Most physicians in the Bitter Root Valley who have had experience with the disease recognize but one type, a severe and usually fatal form, the diagnostic feature of which is the "spots." Several physicians, however, recognize in addition a mild type in which there are no "spots." There is much difficulty in the accurate diagnosis of the mild type, and though its existence must be recognized, yet, during the investigation herewith reported, all of the examinations except one were made on cases of severe type.

CHARTS ILLUSTRATING CASES NO. 96, 116, 122, 124

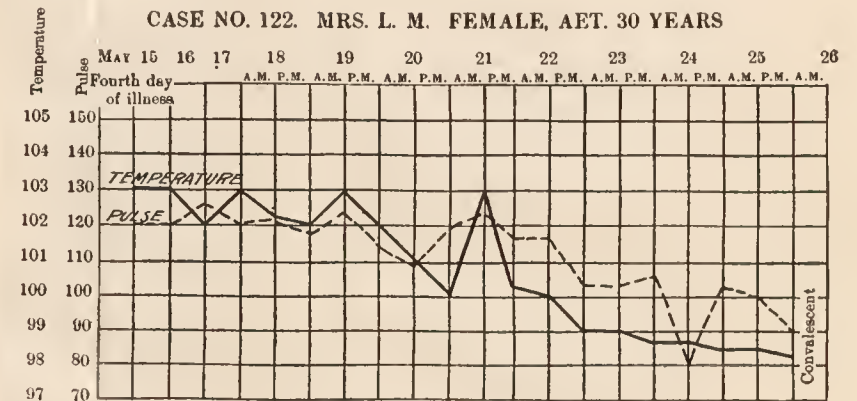
D. McD. (CASE NO. 96.) SHOWING TEMPERATURE, PULSE, AND RESPIRATORY CURVES



CASE NO. 116. MRS. D. FEMALE, AET. 18 YEARS



CASE NO. 122. MRS. L. M. FEMALE, AET. 30 YEARS



CASE NO. 124. O. S. MALE, AET. 33 YEARS

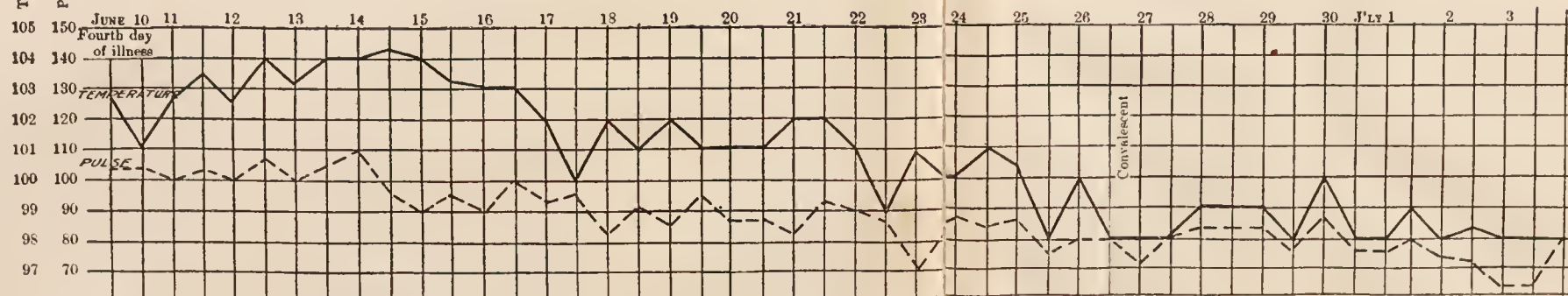


TABLE III.

Showing Red and White Cell Counts and Hemoglobin Estimates in
Pyroplasmosis hominis.

CASE	AGE	SEX	DAYS OF DISEASE						
			2d	4th	5th	6th	7th	8th	9th
94	5	F	4,500,000 12,000 62%						
107	4	M				4,100,000 12,000 50%			Died
116	18	F			4,920,000 7,400 90%		4,600,000 7,600 80%	Died	
117	34	M							4,368,000 7,800 60%
118	40	M					4,576,000 7,300 70%		3,820,000 8,000 50%
120	30	M						4,744,000 4,800 90%	4,722,000 6,900 87%
122	30	F						4,380,000 7,600 75%	4,723,000 10,040 70%
123	31	M							3,408,000 75%
124	33	M		4,100,000 65%					

CASE	AGE	SEX	DAYS OF DISEASE							
			10th	11th	12th	13th	14th	15th	16th	27th
89	34	F	4,200,000 14,000 60%	Died						
94	5	F		Died						
115	8	F						Conva- lescent	4,720,000 4,500 80%	4,824,000 4,450 90%
117	34	M	Died							
118	40	M			3,920,000 8,500 60%		Convalescent on 21st day			
120	30	M	4,720,000 85%	4,458,000 82%	3,558,000 77%	3,672,000 75%	Died			
122	30	F	4,452,000 8,400 66%		60%	3,772,000 62%		4,200,000 62%	Conva- lescent	
123	31	M	Died							

It will be noted that these counts were made in an altitude of about 3,500 feet, where the normal count is above rather than under 5,500,000. The table then shows a marked reduction of red blood-cells and of hemoglobin, with a slight increase of leucocytes at times. The reduction of red cells is particularly marked just before death in fatal cases, and in recovering cases just before beginning of convalescence. One child, case No. 101, of twelve years, examined two months after convalescence, showed Hb. 80 per cent. Freshly drawn blood from patients during their illness when examined with the 1:12 oil-immersion objective shows parasites sparingly in the red blood-cells. Blood was examined from three recovered cases, one of two months, one of one year, and one of two years, without finding the haematozoa. Case No. 115, examined fourteen days after patient had been discharged by physician, still showed parasites present in the blood. The Widal reaction with *B. typhosus* is not present. The spleen is uniformly enlarged and tender on palpation. In all severe cases more or less oedema of the face and extremities is present. This may be marked and may appear as early as the third day of the disease.

Digestive tract.—At the onset of the disease the appetite is usually good, and food is well retained and assimilated. About the beginning of the second week nausea and vomiting develop, and continue in fatal cases to the end. In some cases nausea is present from the onset. Constipation is usually present from the beginning. Abdominal tympanites usually appears one or two hours before death. The liver is somewhat, though not markedly, enlarged. Pain on pressure is absent.

Urinary system.—The urine is reduced to about one-half its normal amount and is slightly above normal in color. A small amount of albumen has been found in each of the five cases examined. Hemoglobinuria is absent or very slight. Both granular and blood casts were present in specimens from each of the nine cases examined.

Respiratory system.—The respiration rate sometimes reaches 60 per minute in the adult, though ordinarily it does not run above 36 per minute. Like the pulse rate, it is frequently out of all proportion to the temperature. It is regular, but usually shallow. It may be labored and accompanied by mucous rales, during the last day or two of life. Cheyne-Stokes respiration has not been observed. Hypostatic pneumonia sometimes develops. Lobar pneumonia occasionally occurs as a complication, and usually hastens the end.

Complications.—The symptoms above noted are sometimes complicated by gangrene, hypostatic pneumonia, articular rheumatism, etc. Two physicians have noted epistaxis, but it has been present in but three of the cases seen by the writers.

PROGNOSIS.

In Montana cases of the mild type of the disease, which show no spots, are as yet too indefinitely differentiated to permit of their inclusion with those of the severe type which invariably develop the eruption. That such cases exist there can be no doubt. They are never fatal. On the other hand, the cases

which are marked by the eruption have a mortality of 70 to 80 per cent. Thus of the 126 cases reported, 88 died and 38 recovered—a mortality of 70+ per cent. If there is omitted from the list 8 early cases, concerning the diagnosis of which some doubt was expressed, and none of which were fatal, the 88 deaths out of a remaining total of 118 cases gives a mortality of 75 per cent.

Death usually occurs between the sixth and the eleventh days. Thus of the 88 fatal cases, death occurred on the third day in 1 case, fourth day in 1 case, fifth day in 3 cases, sixth day in 13 cases, seventh day in 12 cases, eighth day in 13 cases, ninth day in 11 cases, tenth day in 11 cases, eleventh day in 9 cases, twelfth day in 3 cases, thirteenth day in 3 cases, fourteenth day in 2 cases, fifteenth day in 1 case, eighteenth day in 1 case (complication), twenty-seventh day in 1 case (complication second week of convalescence), and on unnoted days in 3 cases. Thus death occurred in 69 cases between the sixth and the eleventh days.

The prognosis of cases in Idaho, Nevada, and Wyoming is much more favorable.

MORBID ANATOMY.

Eight autopsies were made by the writers, in from three to twenty-four hours after the death of the patient.⁷ Four of these were on adult males, two on adult females, one on a four-year-old male, and one on a five-year-old female. The macroscopic lesions were very uniform, and may be summarized as follows: Intense rigor mortis appeared early. The skin over all dependent portions of the body presented a marbled appearance. Over the non-dependent portions it was covered with petechiae. In all cases small wounds of the skin due to tick bites were present. The pleura was normal. The lungs showed hypostatic congestion. The pericardium was normal. The epicardium usually contained a few petechial hemorrhages near the base of the left ventricle. The myocardium was softened. The right ventricle was filled with dark fluid blood: the left was almost empty or contained only a small clot.

The spleen was enlarged, being from three to three and one-half times its normal weight. The capsule was distended and thinned. On section the tissue was found dark red and so soft as to be in most cases diffluent. The outlines of the Malpighian bodies were obliterated. The omentum covering the spleen was usually congested. In some instances the dependent portions of the stomach wall were hyperemic.

⁷ Six were made earlier than eleven hours after death.

The intestines were normal—except slight hypostatic congestion in two cases—throughout their entire extent. The liver was slightly enlarged, pale in color, and of normal consistency.

In all cases one or both kidneys showed small subcapsular hemorrhages on the ventral surface. The capsule stripped readily. The cortex on section was congested. The bladder was normal, and contained a small amount of urine slightly darker than normal. The uterus in the three females examined was apparently normal.

The meninges of the brain and spinal cord showed a slight congestion, apparently hypostatic. There was no basilar (or other) meningitis.

MORBID HISTOLOGY.

(Studies in morbid histology are incomplete. The following is a brief statement of work done.)

Technic.—Specimens at all autopsies were collected from the skin, lung, heart muscle, spleen, liver, and kidney, and from the brain and spinal cord in three instances. These tissues were fixed in 96 per cent. alcohol, 10 per cent. formaldehyde, and Zenker's fluid. After paraffin imbedding, sections 3, 5, and 10 μ thick were cut. Staining was done with hematoxylin and eosin, eosin and Unna's alkaline methylene blue, Nocht-Romanowsky's method, Laveran's method, and Nocard's modification of Laveran's method.

Skin.—The capillaries of the skin are distended with blood which contains an excess of leucocytes. Many red cells have escaped from the vessels into the surrounding tissues. In some cases blood-pigment granules are present in old extravasations. In a few instances phagocytes containing infected red cells are present. Not so many infected red cells are found as are present in the spleen, kidney, and liver.

Lung.—All the lungs show considerable congestion and swelling of the capillaries. Many red blood-cells containing parasites are present. In most of the cases many phagocytes which have taken up infected red cells and pigment granules are found. In one case there is considerable bronchopneumonia.

Heart.—The capillaries of the heart are distended. There is not much extravasation of red cells, but considerable round cell infiltration. All the cases show considerable parenchymatous degeneration. Those cases in which round cell infiltration is marked show also swelling of the muscle fiber nuclei with fragmentation.

Spleen.—The spleen shows an engorgement with red blood-cells and leucocytes. The outlines of the Malpighian bodies are lost. There is a marked infiltration of leucocytes mostly of the polymorphonuclear type in the region of the Malpighian bodies. There is much blood pigment both free and within phagocytes. Many pyroplasmata are present both free and within red cells, many of which have been taken up by phagocytes.

Liver.—The capillaries are distended with blood containing an excess of leucocytes. Many red cells contain parasites. The infected cells are frequently contained within phagocytes. There is an acute parenchymatous

hepatitis, with very marked fatty degeneration. Some of the cases show considerable blood pigment.

Kidney.—The blood-vessels are congested. There is some extravasation of red blood-cells, particularly in the cortex. Many phagocytes containing infected red cells and detritus are present. There is a general acute parenchymatous nephritis.

Brain.—There is a slight congestion of the capillaries of the meninges. A few of the vessels of the cortex contain infected red blood-cells. There is some distension of the pericellular spaces in the cortex. Little or no chromatolysis is shown by Nissl's stain.

Spinal cord.—The vessels are somewhat congested, as in the brain, and contain a small number of infected corpuscles. In one case the anterior horn-cells show considerable chromatolysis with Nissl's stain. No fiber degeneration is shown by Weigert's stain.

Summary.—The changes are those which can be ascribed to interference with capillary circulation. The extravasation into and pigmentation of the skin account for the persistence of the "spots" for long periods after the recovery of the patients. There is acute parenchymatous degeneration of the heart muscle, spleen, liver, and kidney. The central nervous system is but little affected.

ETIOLOGY.

General considerations.—In studying the etiology of this disease the following facts appeared to be of most significance:

1. The disease is definitely limited in locality, being sharply cut off from the east side of the Bitter Root Valley by the Bitter Root River.
2. It is confined entirely to one season of the year, namely, from March to July.
3. It attacks alike patients of any age and either sex, though those whose occupations or pleasures take them to the foothills of mountains in springtime are most affected.
4. All the symptoms and lesions indicate that the disease is due to a specific infection.
5. There is not even a suspicion of its ever having been transferred directly from one human being to another, except in one instance, in which an infant (Case No. 17), born while the mother was suffering from the disease, developed marked purpura on the second day after birth.
6. In no instance have two or more persons with the same food or water supply been simultaneously stricken with the disease.

7. There are no symptoms nor lesions which point to the digestive, respiratory, or genito-urinary tracts as the avenue of infection.

8. In all the cases examined by the writers there were small wounds of the skin, said to have been made by the bites of ticks. In twenty of the cases studied in 1902-3 this history was definite and positive. In three of the cases studied during 1902 and in eleven of the cases studied during 1903 there was a clear history of one or more severe tick bites two to eight days before the initial chill. In most of these cases a continuous chain of local symptoms was present from the date of the bite until the onset of general symptoms.

Bacteria.—In seventeen cases direct coverslip preparations were made from the patient's blood during life. No bacteria were found in any of these. In thirteen of these cases cultures on Löffler's blood-serum, plain agar, and in bouillon were made from the same source as the coverslip preparations, after scrubbing the skin thoroughly with alcohol. Cultures from four of the cases showed a few cocci, which were determined by subcultures to be *Staphylococcus epidermidis albus*. At the eight autopsies—held three, seven, eight, nine, ten, eleven, twenty-two, and twenty-four hours after death, respectively—direct coverslip preparations were made from the subcutaneous tissue, lung, pericardial fluid, heart's blood, spleen, liver, and kidney. In but three autopsies was the central nervous system examined. Here coverslip preparations were made from the meninges and substance of the brain and spinal cord. The coverslip preparations were stained with eosin and Löffler's methylene blue, with eosin and Unna's alkaline methylene blue, and by Gram's method. No bacteria were found in any of them, except a few cocci in one skin preparation (in which case cultures showed the presence of *Staphylococcus epidermidis albus*), and in the preparations from the spleen, which in one case showed a large spore-bearing (putrefactive) bacillus and in another *B. coli communis*. At the autopsies cultures were made from all the sources noted under coverslip preparations—and in addition in three instances from the intestinal contents—on Löffler's blood-serum, nutrient agar, and in bouillon.

About 1 c.c. each of spleen pulp and heart's blood were also sown into flasks containing 250 c.c. each of bouillon. The spleen in five cases, after collection of coverslip preparations, cultures, and histologic specimens (and weighing), was wrapped in gauze, which had been wrung out of 1:1,000 sublimate solution, and incubated for twenty-four hours. Direct coverslip preparations and cultures on Löffler's blood-serum, agar, and in bouillon, were then made from the incubated organ.

All cultures were examined macroscopically and in coverslip preparations stained with Löffler's methylene blue. The bacteria found were as follows:

1. *Staphylococcus epidermidis albus* from the skin in three cases.

2. *Staphylococcus pyogenes aureus* from the skin in one of the above cases.

3. *Bacillus coli communis* was sparingly present in cultures from the spleen in two autopsies. This bacillus was also present, mixed with but few other organisms, in all cultures from the intestinal contents.

4. The spleen from one autopsy showed a large spore-bearing, anaerobic, putrefactive bacillus.

No growth was present except as above noted.

It will thus be seen that no bacteria of etiologic significance were obtained.

Haematozoa.—A few ovoidal bodies were seen within red blood-cells in stained direct coverslip preparations from the second, third, fourth, fifth, and sixth cases examined in 1902. The character of these bodies was not determined, however, until microscopic examination of the fresh blood in the seventh case was made. Similar examinations were made of the fresh blood in three other cases examined in 1902 and nine cases in 1903. In many of the examinations made of the freshly drawn blood, intracellular parasites showing amoeboid movements were found. In several of these observations extracellular forms were found.

In the observations on freshly drawn blood usually several fields were searched before any red cell was found containing a parasite. The same is true of stained coverslip preparations. In

one observation, however, made on the seventh day of illness in an adult male (Case No. 96), on fresh blood many red cells containing parasites were found. In this specimen fields contained from 125 to 175 red cells (estimated). Of these fields two contained eleven infected red cells, each of which held one or two parasites. Another field contained nine infected cells; another seven; and three contained five infected cells each. Nearly every field held one or more infected cells.⁸ Another similar observation on the fresh blood from the same patient, made forty-eight hours later, revealed only a very small number of the infected red cells. In the meantime no material change in the clinical symptoms had occurred.

In a single one of several observations on each of six other cases many infected red cells were found. Three of these observations were made within twenty-four hours of the death of the patient; two of the others were made at periods when the patients were very ill, and the third on the patient's second day of illness, when he was not yet showing severe symptoms.

The parasites may be stained in direct coverslip preparations and in sections by any of the intense methylene blue stains, or by carbol thionin. The results, however, are very unsatisfactory, owing to the light staining of the parasites and the lack of contrast with the other elements. Better effects are obtained with Jenner's and Nocht-Romanowsky's blood-stains. The writers were not able to obtain the ingredients for Laveran's⁹ and Nocard's¹⁰ stains for pyroplasma until October, 1903. These stains have been used on blood preparations and sections collected during the springs of 1902 and 1903. The results are much better than those obtained with any other method, though it has been found necessary materially to increase the relative amount of Borrel blue in Nocard's modification. These are the only methods by which the writers have been able to differentiate the chromatin of the parasite. It is stained red or violet, while the remainder of the organism is pale blue.

⁸The counts in this examination were made after the specimen had been for three and one-half hours under observation at a temperature of about 33° C.

⁹LAVERAN, *Compt. rend. Soc. de Biol.*, 1900, 52, 549.

¹⁰NOCARD, *Ann. de l'Inst. Past.*, 1902, 16, 257.

The organism varies greatly in size, form, and staining reaction at various stages of its development.

The smallest forms (*Phase 1*) slightly resemble "hyaline bodies" of malaria, and very much so *Pyroplasma bigeminum* (see Plate II, Fig. 1). When stained by Laveran's or Nocard's stain, the chromatin masses are seen usually near one end of the parasite. This form is usually paired, and when found free in the blood plasma may be so small as to be mistaken for diplococci. When within the red blood-cells—the usual situation—the parasite is somewhat ovoidal in form and 1μ in thickness and 1 to 2μ in length. Sometimes the organism is roughly spheroidal. While but two parasites are usually present in one red blood-cell, the writers have observed four, six, eight, twelve, and sixteen in a few instances (see Plate II, Figs. 3 and 4). When the organisms are in pairs within a cell their lesser extremities are often toward each other, though distinctly separated by a small interval. The lesser extremities in a few instances have been observed to be joined by a very fine filament. This is true of both intra- and extra-cellular forms (see Plate II, Fig. 5). These small forms have occasionally been seen to change their position within the red cell, though the presence of pseudopodia or any other evidence of amoeboid movement, except the transference from place to place, has not been observed in them.

Several gradations in size, in both single and paired organisms, have occasionally been observed up to *Phase 2*. This phase is marked by an organism usually solitary within the red cell, and ovoidal in form, though it may be elongated, ellipsoidal, or spheroidal. It is from 2 to 3μ thick and 3 to 5μ long. This type in freshly drawn blood frequently exhibits active amoeboid movements. These consist in the elongation of the organism, the projection of pseudopodia in one or more directions, the final retraction of all pseudopodia, and the assumption of the ovoidal form (see Plate II, Fig. 6). One such organism was kept under observation for three and one-half hours. Its movements are shown in the following sketch. (See Fig. 1, p. 48.)

In contrast to the small number of infected cells in the circulating blood is the great number of infected cells in the congested

capillaries in the tissues. This is especially true of the lung, spleen, liver, and kidney. The ovoidal form, however, of most of the parasites appears to be lost shortly after death. In the tissues the parasites contained in phagocytes may be metachromatic to methylene blue. The organisms may be best stained in tissues,

as well as in coverslip preparations, by Laveran's and Nocard's methods (see Plate II, Figs. 7 and 8).

From the above description of the parasite of "spotted fever" it is evident that the organism is a haemocytozoan¹¹ closely related to *Pyroplasma bigeminum*, *Pyroplasma canis*, *Pyroplasma ovis*, and *Pyroplasma equi*. The writers therefore propose placing the organism in the genus *pyroplasma*, designating it *Pyroplasma hominis* Wilson and Chowning. Following this, the term *Pyroplasmosis hominis* would seem to be a desirable technical one for the disease.



FIG. 1.—Showing amoeboid movements of *Pyroplasma hominis* in a red blood-cell. Blood from ear of case No. 96, sixth day of disease. Specimen examined in a room temperature of 80° F. by lamp-light with Zeiss oc. No. 4, Obj. 1-12. Four other r. b. c. out of about 175 in the same field contained parasites. The granule which was first seen at 9:06 P. M. was suddenly extruded from the parasite at 9:57 P. M. and had disappeared from the cell at 9:38 P. M. At 11:30 P. M. the organism was still resting as at 10:58 P. M. (Observations and sketches made by Dr. F. F. Westbrook.)

INOCULATION EXPERIMENTS.

June 4, 1902, about 0.5 c.c. each of the spleen pulp and heart's blood removed at autopsy No. 6 was inoculated, four hours after the death of the patient, into the breast muscles of two pigeons, and also subcutaneously into the right groin of two white rabbits. The blood of the pigeons was examined for several days thereafter, but the organisms were not found. The rabbits, on the day fol-

¹¹ LAVERAN, *Compt. rend. Soc. de Biol.*, 1901, 53, 798.

lowing inoculation, showed a rise of temperature of 1° C. At this time intracellular organisms were found in the blood of both rabbits, and continued present in specimens collected on successive days for two weeks. One of the animals—designated rabbit No. 1—was then killed. At autopsy the spleen was enlarged and darker than normal. It was not softened. There were no macroscopic lesions in any other organ. The tissues were preserved in 95 per cent. alcohol, Zenker's fluid, and 4 per cent. formaldehyde. Examination of these tissues shows many organisms indistinguishable morphologically from those found in tissues from human cases. The second rabbit—designated rabbit No. 2—is still alive, sixteen months after inoculation, and still shows the organisms present, though they are becoming less numerous.

May 12, 1903, two rabbits, Nos. 3 and 4, were inoculated intravenously, one with heart's blood and the other with spleen pulp from case No. 120, twelve hours after the death of the patient (autopsy No. 7). At the same time material from the same sources was inoculated into two Columbian spermophiles. Frequent examinations were made of the blood of all of these animals, but no parasites were found in any of them. This result was probably due to the length of time which elapsed between the death of the patient and the making of the inoculation.

On June 12, 1903, rabbit No. 5, was inoculated intravenously with 20 c.c. of blood drawn two minutes previously from the superficial median vein of case No. 125 (second day of the disease). This rabbit (with rabbit No. 6) was immediately shipped from Missoula to Minneapolis. No clinical symptoms were noted during transit, and on arrival in Minneapolis, June 15, the animal was in apparently good health. On the following four days symptoms of sickness were noted. No temperatures were taken. On June 19 blood from the ear was examined and found to contain many parasites, both intra- and extra-cellular forms being present. On June 20, at 8 A.M., the rabbit died. Autopsy held at 11 A.M. Under the skin, in various places over legs and trunk, were found hemorrhagic areas. The spleen was swollen to about three times normal size. One kidney showed a hemorrhagic condition both on external surface and on section. On June 12, 1903, at 8 P.M.,

rabbit No. 6 was inoculated intravenously with 10 c.c. of blood drawn from the superficial median vein of case No. 124. This rabbit (with No. 5) was shipped to Minneapolis and is still living—apparently well—although frequent examinations have shown the presence of pyroplasmata in the blood. Two sub-inoculations have been made from this animal.

MODE OF INFECTION.

Since there is no suspicion of “spotted fever” ever having been transferred directly from man to man, and since there is no symptomatic or post-mortem evidence of entrance of the disease by way either of the digestive tract, respiratory, or genito-urinary systems, the writers were led to examine the skin for evidence of direct inoculation. In each case under observation, during the investigation, evidence of tick bites was present. But it is true that in the locality in which the cases occur many persons are bitten by ticks and yet show no symptoms of “spotted fever.” However, the following facts suggest the hypothesis that the disease is conveyed to man by means of this arachnid:

1. Ticks appear in the spring as soon as the snow melts from the sunny exposures; *i. e.*, as early as February 15. They are, however, chilled and inactive until the latter part of March. In relation to this, scattering cases of “spotted fever” appear during the latter part of March, and are most numerous during May and June.

2. Ticks become less numerous about the middle of June, and disappear about the middle of July. In connection with this, cases of “spotted fever” become less numerous from the middle of June, and after July 20 no cases have been observed in the Bitter Root Valley.

3. The occurrence of “spotted fever” in isolated cases in a region sharply limited on one side by a river would indicate the conveyance of the germ to man (if by any animal) by a temporarily parasitic animal which travels slowly and not widely, and which is not carried far by the wind, etc. The tick answers this description. Owing to the fact that the valley and foothills of the west side of the river are much more generally wooded than is the

east side, ticks are apparently much more abundant on the west side than on the east side.

4. All haemocytozoa of warm-blooded animals of which the life-cycle is now known pass at least one phase of their development within the body of some host (an insect or arachnid) other than the one whose blood-cells they invade.

5. *Haémamoeba malariae* Laveran, the haematozoan which in man most nearly resembles that of "spotted fever," is conveyed to man by the bite of an insect. *Pyroplasma bigeminum* Smith and Kilborne,¹² the haematozoan which causes "Texas fever" in cattle, and which is apparently a very close relative of the parasite causing "spotted fever," is conveyed to cattle through the bites of ticks of the genus *Boöphilus* (or *Ripicephalus*). *Pyroplasma canis* Piana et Galli Valerio, the parasite causing malignant jaundice in the dog, is conveyed to the dog by the bite of the tick *Haemaphysalis leachi*¹³ in South Africa, and in France and Italy by a tick, *Dermacentor reticulatus*.¹⁴ It is perhaps unnecessary to note that of the many genera of mosquitoes only one carries the malarial organism to man, and of the many genera of ticks only one carries the "Texas fever" organism to cattle; further, that even in these specific genera by no means all of the individuals are affected with the pathogenic parasite.

6. In 1902 the writers thought they had identified three species of ticks in the Bitter Root Valley, namely *Dermacentor reticulatus*, *Dermacentor electus*, and *Ixodes ricinus*. However, specimens were not preserved, and when in 1903 eight lots of ticks were forwarded by the writers and Dr. Anderson from the locality to Dr. Ch. Wardell Stiles, of the United States Public Health and Marine Hospital Service, he at first gave a provisional diagnosis of *Dermacentor reticulatus*. After propagation experiments, however, Dr. Stiles has recently written the authors as follows:

"The ticks are undoubtedly members of the genus *Dermacentor*. They are very closely related to the species *D. reticu-*

¹² SMITH AND KILBORNE, *U. S. Bureau of Animal Industry, Bulletin No. 1*, Washington, 1893.

¹³ ROBERTSON, *J. Comp. Path. and Therapeut.*, 14, 327; and LOUNSBURY, *Veterinarian*, 75, 86.

¹⁴ NOCARD AND MOTAS, *Ann. de l'Inst. Past.*, 1902, 16, 257.

latus, but I have noticed certain differences which I am inclined to believe extend beyond the limit of specific variations, and which I think will eventually justify the erection of a new species for the ticks in question. I hesitate, however, to propose a new species until I obtain some more specimens, and also until I am able to compare them with further material of *D. reticulatus*. The most constant difference between the specimens from Montana and my material of *D. reticulatus* is in the stigmal plate. Should an examination of a large number of specimens show that the characters observed in the stigmal plate are constant, I think there will be no doubt regarding the validity of a new species."

It is perhaps worthy of note that *Dermacentor reticulatus* is the species described by Nocard and Motas¹⁴ as conveying *Pyroplasma canis* to dogs.

7. Dr. H. P. Johnson¹⁵ during 1902 and 1903 has made an investigation for the Montana State Veterinary Department of a disease of sheep in the Deer Lodge Valley, in which he has demonstrated that the disease in question is pyroplasmatic icterohaematuria caused by *Pyroplasma ovis*.

8. All of the patients—twenty-three—coming under observation during this investigation had been bitten by ticks. In fourteen cases a history was given of severe tick bites two to eight days before the onset of the disease. In a number of other cases an apparently clear history of severe tick bites immediately preceding the onset of "spotted fever" was vouched for by the recovered patients or their friends.

9. In the case of W. G., reported in 1902, a local infection with the presence of haemocytzoa morphologically identical with those present in cases of "spotted fever" was certainly induced by the bite of a tick.

10. There is apparently no other insect, arachnid, or other biting creature within the infected locality which fulfils the conditions indicated in the above outline as does the tick.

¹⁴ *Loc. cit.*

¹⁵ JOHNSON, *Proc. Am. Vet. Med. Assoc.*, 1903.

SPERMOPHILUS COLUMBIANUS POSSIBLY ALSO A HOST OF PYROPLASMA HOMINIS.

The extreme isolation of cases of "spotted fever," their occasional development in localities removed many miles from the site of any previous case, and the long period existing between the death or convalescence of the last case of any one year before the development of the first case in the following years, render improbable the invariable transference of the haemocytzoa even indirectly—*i. e.*, via an arachnid—from previous cases to new ones. The early recognition of this fact led the writers to consider the possibility of the red blood-cells of some one of the lower warm-blooded animals being the normal habitat of the pyroplasma in that stage of its life-cycle not passed within the body of some arachnid. They are aware that such a multiplicity of hosts of any of the haemocytzoa has not been established, yet such a possibility is suggested by a careful study of the cases of "spotted fever." The probability of any species of bird serving as such a host is rendered slight by the unique geographical distribution of the disease. Of the mammals of the locality the only one whose geographical and seasonal distribution bears any apparent relationship to the disease is *Spermophilus columbianus*, a southern relative of Parry's Arctic spermophile.

The writers gathered specimens from various animals in both uninfected and infected areas. Blood-smears from seven horses from an infected area were examined. All the other animals examined were rodents, and particularly the above mentioned spermophiles. Specimens were collected from rodents in the following areas (see map opposite p. 33):

1. *Uninfected areas*.—(a) On the east side of the Bitter Root River, beginning at the Hellgate River and extending to a point about eight miles south of Stevensville, a distance of about forty miles, and including the valley, from the river bank to the upper foothills ten miles east of the river, near the head waters of Burnt Fork Creek. (b) On the north side of the Hellgate River, in the neighborhood of Grant Creek, three miles west of Missoula. (c) On the north side of the Hellgate Rive, one mile east of Missoula.

2. *Infected area*.—On the west side of the Bitter Root River, from near its mouth to a point about three miles south of Hamilton, and from its banks to near the summit of the Bitter Root range of mountains (on Mount Lo Lo). The first west-side spermophile examined was killed within thirty yards of the house where case No. 95 was lying sick. A number of spermophiles were killed near Florence, in the vicinity of which many cases of "spotted fever" have occurred. A number were killed near the sites of cases Nos. 89, 115, 116, 119, and 120.

The hemoglobin estimations of spermophile blood from both the infected and uninfected areas average between 90 and 100 per cent. (Tallquist), and the red cell counts average 8,418,500, the counts ranging from 7,264,000 to 9,218,000. The results of the examination of the blood of the spermophiles and other rodents for parasites were as follows:

1. No parasites were found in the blood of any animal except spermophiles. The list examined includes horses, pine squirrels (*Sciurus hudsonius* var. *richardsoni*), chipmunks (*Tamias asiaticus*), woodchucks (*Arctomys flaviventer*), and one "snow shoe" rabbit (*Lepus Americanus*?). Besides these animals the blood of over one hundred well persons, or persons suffering from a variety of other diseases, was examined, with negative results.

2. There was found in the blood of a few spermophiles from both infected and uninfected areas two large parasites, both outside the red cell.

3. Ten of the fifty-one spermophiles from the west—infected—side of the valley showed within their red blood cells haemocytozoa which in size, shape, staining reactions, and movements (where fresh blood was examined) were indistinguishable from the haemocytozoa found in the blood of patients affected with "spotted fever."

4. Blood and tissues have been examined from sixty-two spermophiles from the east—uninfected—side of the valley, and from other near-by uninfected localities, without the discovery as yet of any haemocytozoa similar to those found in spermophiles from the infected area. This negative evidence must, of course, be more extensive before much weight can be attached to it.

The following is a brief summary of the facts which seem to be of importance in considering the possibility of *Spermophilus columbianus* being a third host of *Pyroplasma hominis*.

1. *Spermophilus columbianus* has but a limited distribution in the United States. This is in the northern Rocky Mountain region within which lies western Montana.

2. It is very abundant on the west—infected—side of the Bitter Root Valley. It is much less abundant on the east—uninfected—side. This was certainly true during the spring of 1902, as was evidenced by the few colonies discoverable on the east side. Residents of the district agree that this is usually the case.

3. In the Bitter Root Valley the animal lives in rather sharply defined colonies, and is not much of a wanderer from home except at the rutting season.

4. While the animal is able to swim, it is averse to doing so. During the spring months the Bitter Root River is swollen with rains and melting snows until it becomes a wide and swift torrent. There are few bridges, and it is difficult to conceive of any general passing back and forth to the two sides of the valley by the spermophile.

5. Local accounts disagree as to the date when the spermophile emerges from hibernation, but it is probably about March 1–15.

6. Local observers generally agree that in the Bitter Root valley the spermophiles begin hibernating about August 15.

7. Early in the spring the spermophile is said to harbor great numbers of ticks. At the time the writers were making their investigation—April to July—very few ticks were found attached to spermophiles.

8. Twenty per cent. of the spermophiles examined from the infected area had in their red blood-cells haemocytozoa indistinguishable morphologically from those found in the red blood-cells of patients suffering from "spotted fever."

9. No similar parasites have been found in the blood of any of the sixty-two spermophiles collected in uninfected, though near-by, localities.

10. No similar parasites have been found in the blood of any other animals, nor in the blood of any one of the many healthy

persons or persons sick of other diseases examined in the same locality.

11. That it is possible for one rodent—the rabbit—to carry within its red blood-cells for a long time the pyroplasmata of “spotted fever” without the exhibition of any symptoms has been shown by the inoculation experiments noted on p. 48. The spermophile may be similarly unaffected by, though infected with, the parasites.

DIAGNOSIS.

The diagnosis of *Pyroplasmosis hominis* early in the history of the case is sometimes extremely difficult or impossible. The prodromal symptoms are not unlike those of influenza and typhoid fever. When, however, in the spring and early summer months in the infected locality patients present a history of constipation, headache, pains in back or limbs or radiating from a tick bite, muscular soreness, chill followed by rise of temperature, and usually, mental apathy, there is good reason to suspect the presence of the disease. Where in addition to these symptoms a rash appears first on the extremities, usually on the third day after the chill, and becomes petechial in character, or where a blood examination reveals the presence of *Pyroplasma hominis*, the diagnosis is positive.

PREVENTIVE MEASURES.

In view of the almost certain rôle of the tick in the conveyance of pyroplasmosis to man, measures should be taken to reduce the numbers and limit the spread of this arachnid. The burning of underbrush, sawdust, etc., wherever practicable, is recommended. Persons going into the brush in the infected area should use all possible precautions to prevent ticks from biting them. As soon as a person is bitten by a tick, the arachnid should be removed and the wound cauterized with 95 per cent. carbolic acid.

TREATMENT.

Many drugs have been used in the treatment of “spotted fever,” but while some of them are important as stimulants, sedatives, etc., none of them—except perhaps quinine—seem to have

any specific action on the disease through destruction of the parasite. Quinine has been used by the physicians of the Bitter Root Valley in small doses by mouth for a number of years in the treatment of the disease. During the spring of 1902 the writers urged, on purely theoretical grounds, the use of large doses of the drug intravenously, hypodermically, or per rectum. Cases Nos. 94 and 95 were given quinine by mouth and rectum to the point of cinchonism, with some apparent beneficial results. Both cases died, though No. 95 was convalescent from "spotted fever" and died of a complicating pneumonia. During 1903 cases Nos. 115, 118, 119, 122, 124, 125, and 126 were treated with doses ranging from 5 grains by mouth (case No. 119) to 60 grains subcutaneously (case No. 118). All of these cases, except No. 125, recovered, though case No. 119 died later of a complication (acute gastritis). Case 125 was an old debilitated man, and had the most abundant infection of all cases examined by the writers. The remaining five cases occurring in 1903 and untreated with quinine were all fatal. These cases are too few on which to base conclusions, but are sufficiently suggestive to warrant a further trial of the treatment. In this connection it is worthy of note that Theiler¹⁶ has recently made the observation that in South African equine malaria—a disease caused by *Pyroplasma equi*—the pyroplasmata rapidly disappear from the blood of infected horses on the administration of quinine and ammonium chloride.

Darkening of the room and hot sponge baths add much to the comfort of the patient.

MINNEAPOLIS,
October 15, 1903.

NOTE.—Since this article was placed in the editor's hands, Bulletin No. 15, U. S. Public Health and Marine Hospital Service, has appeared, entitled *Spotted Fever (Tick Fever) of the Rocky Mountains. A New Disease*. By Dr. John F. Anderson. Reproductions of our map, two of our photographs, and our notes on cases Nos. 115–119 inclusive, through oversight unacknowledged, are included in this Bulletin.

MINNEAPOLIS,
December 9, 1903.

¹⁶ THEILER, *Jour. Comp. Path. and Therapeut.*, 16, 97, 1903.

A REPORT ON TWO CASES OF A PECULIAR FORM OF HAND INFECTION

DUE TO AN ORGANISM RESEMBLING THE
KOCH-WEEKS BACILLUS.*

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CLINICAL HISTORY.

TWO CASES of acute finger infection, one accidentally inoculated from the other, having certain unusual clinical characteristics, have come under our observation. The signs and symptoms of these cases differed so much in some respects from the ordinary inflammations of the coverings of the hand that the pathogenic organisms present were carefully investigated.

Case I occurred in Dr. W., a hospital surgeon, his account of it and the treatment by himself and others being as follows:

During April he had been treating cases of acute suppurating conjunctivitis and one case of leg infection which ended in septicemia. On April 22, three days after an operation on the septicemic case, Dr. W. noticed a small, painful, reddish papule on the tip of his right forefinger. This increased a little in size, and about four days later the papule was incised. No pus was found. The swelling increased, and two days after the incision the finger was again lanced on April 28, by a nurse, Miss B., who pricked her own finger during the operation.

The secretion from the wound was comparatively clear and serous in character. The edges of the incision formed an elevated, pinkish, granular, cauliflower-like mass of tissue.

About the end of the second week the pain became so severe that it interfered with sleep, and the whole wound was thoroughly curetted.

By the end of the third week the cauliflower-like tissue had reformed, and the pain increased again, the whole finger becoming greatly swollen at the same time. Pus was now detected in the first proximal phalanx. This was opened. By the twenty-eighth day pus had formed on the dorsal surface of the hand between the first and second metacarpal bones. During this

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time the other fingers did not swell, but the whole body of the hand was swollen to about twice the normal size.

From this time on the discharge and pain steadily decreased with the swelling. In the course of about four weeks finger motions gradually returned. The temperature ran a rather irregular course, being highest,



FIG. 1.—Dr. W.'s hand. The photograph was taken at a time when retrogressive changes had set in. At the tip of the forefinger some remains of the cauliflower-like granulations may still be seen. (Photo. by Martin.)

102° F., on the seventeenth. About the twenty-first it reached normal. The pain was intense and not affected by heat or cold. There was great mental depression and complete loss of appetite. Dr. W. lost thirteen pounds in weight. The general course of treatment was a haphazard use of bichloride dressings, 5 per cent. carbolic acid baths, or constant immersion in saturated acetate of aluminum and peroxide of hydrogen.

Case II.—On April 28, 1903, Miss B., hospital nurse, while making an incision into Dr. W's finger for a painful papule, accidentally pricked the pulp of the index finger of her left hand with the point of the knife used. Some bichloride of mercury solution was immediately applied and followed by 95 per cent. alcohol compresses. No pain was felt in the minute puncture, and after twelve hours the compresses were removed.

Thirty six hours later a fullness and throbbing began in the end of the finger, and at the end of another twenty-four hours a small, painful, intracutaneous vesicle appeared at the site of the knife wound. This was opened under antiseptic precautions, and a fraction of a drop of clear fluid liberated. The minute cavity was freely exposed, and a saturated solution of acetate of aluminum was kept in contact with the opening, and with the entire finger as well, for four days. No relief was experienced. Upon changing the dressings daily, a slight staining, equivalent to two or three drops of wound secretion, was noticeable. No redness or swelling appeared.

Throughout the second week incessant stabbing pains were experienced in the finger. The digital arteries pulsated with a wire-like tension. The sensation of painful throbbing and fulness gradually increased; but still no redness or swelling of the finger appeared, nor was there local or general rise of temperature.

The general condition of the patient, up to this time, was that of pronounced mental and physical prostration, vigilance, sleeplessness, and anorexia with vomiting. Some rest was secured through the exhibition of large doses of trional or hypodermatic injections of morphine.

By May 12, fourteen days after infection, the lesion had grown to a diameter of 6 or 8 mm., was circular, and had a soft, non-bleeding, slightly elevated reddish surface, which produced a small amount of secretion daily. On this date, May 12, a general anesthetic was given and the well-like lesion thoroughly curetted. The small curette fell almost of its own weight through the granulations, which seemed filamentous, and attached by one end to the bone, and could be waved about by the instrument, which came at once upon an exposed bony surface of the terminal phalanx. The curetted wound was swabbed dry with gauze, 95 per cent. carbolic acid carefully applied to the cavity, and the finger covered with a mild wet mercuric chloride dressing. Complete relief was experienced for thirty-six hours; then the former symptoms returned with increased severity.

On the sixteenth day, under local anesthesia, the curetting was repeated and a wet dressing of saturated solution of acetate of aluminum applied. No relief resulted, and about the eighteenth day some swelling of this finger, the two adjacent fingers, and of the back of the hand began, and the painful symptoms became even worse.

The patient was again given a general anesthetic on the twentieth day. The terminal phalanx was perforated at its center with a very small ear gouge, with the hope of relieving a pain of central osseous origin. The distal interphalangeal articulation did not seem to be infected. Then an incision 2 cm. long was made over the swollen center of the first phalanx on its palmar surface, and carried down to and into the sheath of the flexor profundis tendon. Through this opening an irrigating fluid passed readily out

at the defect in the end of the finger. A localized swelling on a line with the index finger in the palm of the hand was also incised and found to be principally an edema, but a small circumscribed collection of turbid fluid was also found.

A constant irrigation of cold saturated solution of acetate of aluminum



FIG. 2.—A photograph of Miss B.'s hand, taken at a time when gangrene of the fore-finger had set in. (Photo. by Martin.)

was kept moving through the finger wound along the course of the tendon sheath for about four days.

By this time the third and fourth fingers and the entire hand had swelled considerably, and evidences of gangrene of all tissues of the end of the finger were apparent. For three days previous to the appearance of this tissue necrosis the patient had kept the entire hand in a basin of ice-cold aluminum-acetate solution, in addition to the constant irrigation, because the cold immersion gave some relief from pain.

On June 2 chloroform was given and an atypical amputation made. The terminal phalanx which was lying loose, and the distal articular end of the second phalanx, were sacrificed. Upon drawing upon the long flexor tendon, nearly three inches of necrotic tendon pulled out easily. Healing progressed favorably.

From May 15 to May 25 there were occasional rigors, and the afternoon temperature twice reached 102° F., but usually ranged between 99° and 101°. During this period nutrient enemata with brandy were necessary.

SOME GENERAL CONSIDERATIONS OF PANARIS.

Acute inflammations of the soft parts of the hands are of as much more importance than those of the feet as the usefulness of the hand exceeds that of the analogous parts of the foot. Surgeons treat all these inflammatory processes, usually described as panaris or panaritium, as if a disease of one causative origin, and direct all efforts to preserve the functional uses of hand and fingers. It may be questioned if this is in accord with the present state of our knowledge of these conditions, but actual results bear out the general practice in a striking way, and will continue to do so until specific agents have been produced with which to combat the special pathogenic microbes of these inflammations.

König's conclusion still holds good, that "scarcely in any other of the daily occurring diseases do such bad results follow neglect, or are such favorable functional issues obtained from correct treatment, as in panaritium."

How much can be preserved here by care and skill, and how much lost of the capital of existence by neglect!

Every panaritium is the expression of a traumatic inflammation, whether we are able to discover the point of entrance of the infective matter or not.

Koch's culture experiments have shown that in every case one kind of germ or another, or several together, can be found in the inflammatory exudate, and they are usually briefly described as *pyogenic bacteria*. Streptococci, staphylococci, and in rare exceptions bacilli, such as *Proteus* and *Coli communis*, have been found and the clinical course in all cases seems much alike and to be modified similarly by the anatomical peculiarities of the part first affected.

The usual history of extension of the destructive processes along the paths of least resistance has given rise to the imperative therapeutic law: incision, disinfection, and drainage as soon as possible, and let the cuts be made too soon and too deep and long rather than too late.

Severe pain is usually explained by interference with circulation as a result of local pressure from swelling and its attendant tissue necrosis, and thus the usual panaris resembles in many ways a furunculous inflammation. A second phenomenon which often surprises the novice is the early appearance of redness and swelling of the dorsum of the hand when the primary focus of infection is beneath the volar surface, and where eventually the pus is almost always found. This has its practical significance, and its pathogenic and anatomic causes are too well understood to need description here.

Case II particularly differed clinically, it will be seen, from the usual finger and hand infection in the slowness of the infective process, the absence of pus formation, and the invariable accompanying redness and swelling, in the earlier stages. Then also the severity of pain and physical prostration seemed out of proportion to the extent of the lesions. The course of the infection was determined, as in any panaris, by the anatomical peculiarities of the tissues successively involved. The most striking feature of the treatment was, perhaps, the total failure favorably to influence the progress of the disease by ordinary approved surgical measures. The necrosis of half of Miss B.'s finger was more extensive than was expected, although few cases escape without any tissue loss in severe inflammatory affections of these parts. It is probable that the prolonged immersion of the hand in the ice-cold antiseptic solution had something to do with reducing the vitality of the tissues, and contributing to this result.

BACTERIOLOGICAL EXAMINATION.

Coverslip preparations of the muco-purulent discharge from Dr. W.'s and Miss B.'s fingers show numerous leucocytes and mucous threads. Occasionally a cell is seen containing a number of thin influenza-like bacilli (see Fig. 3). Some of these organ-

isms lie without the cells, singly and in groups. The rods often lie side by side, or may occur end to end, in pairs or short chains. Sometimes they are so short as to appear coccoid. In the preparations from Miss B.'s finger the bacilli are much more numerous than in those from Dr. W.'s finger, often lying outside of the



FIG. 3.—Drawn from a coverslip preparation from Miss B.'s finger. It shows a polymorphonuclear phagocyte containing numerous bacilli (enlarged to show the morphology and irregular staining of the rods). The small figure to the left shows another cell from the same preparation as seen with the Zeiss oil im. 1/12, oc. 3.

leucocytes in masses or groups of a hundred or more rods, or again in short chains of eight or ten bacilli. They stain well with carbol thionin or carbol fuchsin diluted 1:10. Often they take the stain evenly, but may appear segmented, or stain more intensely at the poles, especially when carbol thionin is used. They do not retain the stain in Gram's method. When colored with hot carbol fuchsin, they lose the dye when $\frac{1}{4}$ per cent. acetic acid or ordinary alcohol is applied. In size they vary from about $\frac{1}{2}$ to 3μ in length, by less than $\frac{1}{2}\mu$ in breadth (Zeiss oc. micron).

Throughout the acute stage of the infection this bacillus was present in considerable numbers, and apparently in pure culture. When the acute symptoms had subsided, it could be found only with difficulty, and finally disappeared. After gangrene appeared in Miss B.'s finger, nothing but *staphylococcus pyogenes albus* could be isolated.

Numerous attempts at cultivating the bacillus were made, and at first without success. Inoculations of the secretion upon plain agar¹ slants, agar smeared with human blood, ascitic fluid agar, and glucose agar, grown aerobically and anaerobically (pyrogallic acid method), remained sterile during ten to fourteen days' observation.

¹ The "ordinary" media used were prepared according to the directions of the American Committee (*Reports and Papers of the Am. Pub. Health Assn.*, 1898, 23, 60), with a reaction of 1 per cent. acid to phenolphthalein. The ascitic fluid agar was kindly furnished by Mr. Lindquist, of the First Reserve Hospital Laboratory. It was prepared by mixing equal parts of fluid glucose agar and ascitic serum, and then sterilized by the intermittent method. For better methods of preparing human serum media see EYRE, *J. of Path.*, 1900, 6, 6, and LIBMAN, *N. Y. Med. Rec.*, 1903, 63, 237.

Finally, pieces of tissue removed in an aseptic manner from Miss B.'s finger were forced into the substance of a slant of ascitic fluid agar and incubated at body temperature. No growth appeared during the first three days' incubation, but on the fourth day one of the pieces of tissue was surrounded by a halo of cloudiness, which gradually increased in density and finally assumed a yellowish color, especially near the surface of the slant. Transplants upon ascitic fluid agar showed no growth in twenty-four hours, but in forty-eight hours a delicate, typhoid-like, whitish growth could be seen along the line of inoculation. This gradually assumed a lemon-yellow color. Stained preparations from this growth showed small, slender rods resembling those seen in the preparations made directly from the purulent exudate. When suspended in a hanging drop of bouillon, these rods were very actively motile. They lost the color when Gram's method was applied.

Notwithstanding the difficulty experienced in obtaining the first culture, the bacillus adapted itself to a saprophytic existence and grew rapidly upon the "ordinary" media. In no instance could growth be detected with the naked eye after twenty-four hours' incubation, but it was evident in forty-eight hours. (This peculiarity persisted in ten or twelve transplants during two months' cultivation, but after about three months of saprophytic existence the growth, though faint, is visible in twenty-four hours.)

Cultural characteristics.—On a slant of nutrient agar the growth is visible in forty-eight hours as a distinctly yellowish streak, confluent below, with small, circular, isolated colonies above, and much more luxuriant than that upon ascitic fluid agar. In a stained preparation the bacilli appear thicker than when taken from ascitic fluid agar and show a considerable change in their morphology, most of them being spherical or coccoid, others ovoid and in pairs. A few appear as distinct rods. They are non-motile when suspended in a drop of bouillon. When an isolated colony from such an agar slant or from an agar plate is transplanted upon ascitic fluid agar, the growth reverts in its appearance to the more delicate type, while the organisms all assume the thin bacillary form—actively motile when suspended in a drop of bouillon.

When plated in agar, pin-point-sized colonies are visible in forty-eight hours, and in four or five days reach a diameter of 1-2 mm. In forty-eight hours the superficial colonies appear, under a low-power lens, as uniform granular disks with translucent edges. In four days coarser granulations may be seen about the central portion of the disks, and the colonies assume a chrome-yellow color. These colonies, situated between the agar and the Petri disk, spread out like the superficial colonies, and have the same appearance microscopically, but are unpigmented.

In bouillon a faint, uniform cloudiness appears in forty-eight hours. In the hanging drop short non-motile rods may be seen. In a week the fluid is densely clouded, a sediment collects at the bottom of the tube, while a yellowish-white pellicle is formed on the surface. This pellicle, if unbroken, becomes thick and wrinkled in the course of ten or twelve days. No indol is produced.

In Dunham's peptone solution a faint turbidity appears in four days. No pellicle is formed, and the growth is gradually precipitated. No indol is produced.

In nutrient gelatin (20 per cent., liquefied by the warm weather) growth appears as small isolated colonies floating on the surface and scattered throughout the medium. No peptonization occurs during five days' growth, at about 28°-30° C., for the gelatin solidifies readily when placed upon ice.

In litmus milk no visible change is produced during the first four days. In a week there is a slight reduction of the litmus at the bottom of the tube. In twelve days the litmus is completely reduced, excepting at the surface, where it is still blue for a depth of 2 or 3 mm. No coagulation occurs.

In stab cultures in glucose agar and litmus lactose agar no particular changes are produced. A granular growth appears along the upper portions of the stab, while on the surface a smooth, circular, elevated growth appears. This gradually changes in color from a lemon to a chrome yellow.

In glucose bouillon in the fermentation tube no gas is produced, and the closed arm remains clear during twelve days' observation.

On a slant of potato the first growth appears in four days as minute lemon-yellow colonies. In eight days the colonies are confluent, of a chrome-yellow color, and present a wrinkled surface. In ten days the surface is elevated into snake-like folds. When stained from an eight-day potato culture, the bacilli appear as irregularly clubbed rods. Nothing resembling spores can be stained.

INOCULATION EXPERIMENTS.

1. Monkey² No. 260 received a subcutaneous injection in the chest of $1\frac{1}{2}$ c.c. of a physiological salt solution emulsion of the purulent secretion from Miss B.'s finger. No result followed. A few days later a small piece of tissue curetted from Miss B.'s finger was placed under the skin of the chest and the wound covered with flexible collodion. Four days later a small amount of purulent secretion appeared through cracks in the collodion dressing. This contained pus cells, and a few small bacilli which could not be grown upon plain agar. In a few days the wound had healed completely.

2. Monkey No. 261 received a subcutaneous injection in the chest of $1\frac{1}{2}$ c.c. of a well-clouded emulsion of the purulent secretion from Dr. W.'s finger. Microscopical examination of the pus showed that numerous bacilli were present. In forty-eight hours a pea-sized nodule appeared at the site of injection. Two days later this had completely disappeared, and the monkey remained well during a month's observation. Then it received in the tip of the right forefinger an injection of $\frac{1}{2}$ c.c. of a twenty-four-hour bouillon culture of the bacillus isolated from Miss B.'s finger. Microscopically the culture showed numerous coccoid bodies and short rods. No particular change was noticed during the first two weeks' observation, but in the course of a month the monkey's hand showed an inflammatory process affecting the first three fingers. The tip of the forefinger was gangrenous, and the middle and ring fingers showed swollen, globular, whitish tips. Coverslip preparations showed cocci and bacilli; many of the latter were very minute rods, losing the stain when Gram's

² The monkeys used in these experiments were the common red monkey of the Philippines.

method was applied. They could not be isolated upon ordinary agar plates, and plates made with human serum, according to the method of Pakes,³ were rapidly overgrown by other organisms. The staphylococcus aureus and albus and some unidentified, large, rapidly growing bacilli were present in considerable numbers. The animal was chloroformed, and at the autopsy nothing in particular was noticed, excepting swelling and congestion of the right axillary glands. Sections from the spleen, liver, and kidney appeared normal. Those from the right forefinger showed that the inflammatory process was apparently limited to the outer layers of the skin, the malpighian layer being almost wholly replaced by a mass of nuclear fragments and many cocci and bacilli. The interpapillary pegs of the epidermis showed marked canthosis, long, slender processes of epithelium running deep into the subcutaneous tissue. The subcutis showed almost no infiltration.

3. A young rabbit, No. 269, received in the subcutaneous tissue of the left ear an injection of about $\frac{1}{8}$ c.c. of a densely clouded, seven-day-old bouillon culture of the bacillus isolated from Miss B.'s finger. This was followed by hyperemia at the site of the injection, but no inflammatory process resulted.

4. A young rabbit, No. 272, received an intravenous injection of $\frac{1}{2}$ c.c. of an emulsion of the same bacillus taken from a seven-day-old culture on ascitic fluid agar. It remained well during two weeks' observation, when it escaped.

5. Guinea pig, No. 270, received an intraperitoneal injection of 1 c.c. of a seven-day-old bouillon culture of the same bacillus. It remained well during a month's observation.

RESULTS OF THE AGGLUTINATION TEST.

Blood for these tests was obtained from Dr. W.'s ear and from Miss B.'s infected finger at the time when the infectious process was in a retrogressive stage. Both specimens failed to agglutinate the bacillus, in two hours, at a dilution of 1:10.

There were several cases of acute contagious conjunctivitis in the hospital at the time when Dr. W.'s finger was infected. The

³ See EYRE, *loc. cit.*

bacilli in the coverslip preparations from the cases correspond in their morphology, size, and staining reactions with those in the preparations from Dr. W.'s and Miss B.'s fingers. They also correspond in their morphology, distribution, and staining reactions with the descriptions of the Koch-Weeks bacillus. They could not be cultivated upon the ordinary media nor upon agar smeared with human blood. As we believe the organism described above to be the Koch-Weeks bacillus itself, or a closely related "type" of this organism, it seems desirable to append a description of this bacillus and of other organisms described as resembling it.

Acute contagious conjunctivitis (otherwise known as acute or epidemic catarrhal conjunctivitis, muco-purulent conjunctivitis, or vulgarly as "pink-eye") is a highly contagious, muco-purulent inflammation of the conjunctiva, accompanied by some swelling of the lids (Weeks). The disease is self-limited, passing through an acute stage and then gradually subsiding. Recovery usually occurs in from two to three weeks. The disease is said to be especially prevalent in Egypt, but it occurs throughout the globe.

The specific cause of the disease, a small bacillus, was first seen in a case of conjunctival catarrh by Koch⁴ in Egypt (1883), and cultivated and proved to be the specific micro-organism by Weeks⁵ in America (1886). Therefore the virus is usually known as the Koch-Weeks bacillus. Their work has since been confirmed by Kartulis⁶ in Egypt, Morax in France, and Wildbrand, Sanger and Stalin⁷ in Germany.

The Koch-Weeks bacillus is described as a minute, non-motile rod ($0.25 \times 1\mu$), resembling the influenza bacillus or that of mouse septicemia. In acute cases the bacilli occur in large numbers in the muco-purulent secretion, often being taken up in considerable numbers by the phagocytes. In subacute cases they may be scarce, and in the later stages of the disease they disappear entirely. They often lie in pairs, end to end, or sometimes in short chains within and outside of the pus cells of the secretion. They stain well with the ordinary dyes, but do not retain the stain when Gram's method is applied.

When transferred from the human body to artificial media this organism is even more restricted in its conditions of growth than the influenza bacillus, as it rarely grows upon agar smeared with human blood, the best medium being human serum agar. According to Kartulis:⁶ "When incubated at a temperature between 28° and 30° C., the growth is first visible to the naked eye in a period of thirty to forty hours. At first it appears along the line of inoculation as small grayish-white points. Gradually the points unite into a

⁴ S. GAFFKY, *Arbeiten aus dem kaisert. Gesundheitsamte*, 3.

⁵ *Archiv. of Ophthalm.*, 1886, 15, No. 4; or *N. Y. Med. Rec.*, May 21, 1887.

⁶ *Cent. f. Bakt. u. Parasitenk.*, 1887, 1, 289.

⁷ *Jahrb. d. Hamb. Staatskrankenanstalten*, 3, 94.

small streak, which is raised considerably above the surface of the medium. At this time the culture becomes glossy and darker in color. The borders are uneven and well formed, and very much indented. For a long time the growth retains the described form, but it dries gradually, and then resembles the backbone of a fish. Growth upon agar-agar and gelatin is feeble. Gelatin is not liquefied. Microscopically, in a very young culture the bacillus appears somewhat plumper than those in the cells; in a very old culture they are somewhat longer and thicker. Spore formation has not been observed."

Pathogenicity.—"Transmission to the conjunctiva of animals is unsuccessful. Kartulis has obtained positive results in human beings in only one case out of six, and that with cultures ten to twenty generations old. Weeks, on the other hand, reports several successful inoculations into human beings. His cultures, however, were not pure, but contained, besides the conjunctivitis bacillus, *Xerosis bacilli* (diphtheria group)."⁸

Other organisms described as resembling the Koch-Weeks bacillus.—"Another organism exceedingly like the previous, apparently differing from it only in the rather wider conditions of growth, is Müller's bacillus."⁹

"Bacillus pseudoconjunctivitis".⁸—Discovered by Kartulis in the conjunctival secretions in a case at Alexandria and turned over to the author. These bacilli are non-motile and just as small as the preceding ones, and likewise neither stain according to Gram nor form spores. Their cultures are rather prolific, with a canary-yellow pigmentation. The gelatin cultures liquefied first, although quite slowly, but later the liquefaction disappeared, and the needle-point cultures assumed the form of a nail with flat, canary-yellow heads. On potatoes a well spread out, light-brownish deposit was formed.

"Bacillus aeris minutissimus".⁸—Was obtained by Drs. Jbrahim Bey and Faud Bey in the air at the Institute of Hygiene in Bonn. It is similar to the preceding ones, but forms a light yellowish pigment. It is not pathogenic for animals.

"Bacillus aureus minutissimus".⁸—Also isolated by Jbrahim and Faud on plates exposed to the air. Morphologically similar to the preceding ones, but motile; does not stain according to Gram; does not form spores; liquefies gelatin. On potatoes it forms a luxuriant golden pullulation. It produced septicemia in mice and abscesses in rabbits."

We are inclined to consider the organism isolated from Miss B.'s finger as identical with, or as a closely related type of, the Koch-Weeks bacillus. In addition to its similar morphology, the difficulty with which it was cultivated and the characteristic delay in the appearance of the growth speak for such an identity.¹⁰ We

⁸ FLÜGGE, *Mikroorganismen*, 1896, 2, 440 and 441.

⁹ MUIR AND RITCHIE, *Manual of Bacteriology*, Am. ed., 1903, 201 (MÜLLER'S article will be found in the *Wien. Med. Wchnschr.*, 1897, Bd. 47).

¹⁰ The only literature within our reach is that in FLÜGGE, *Mikroorganismen* (5), and an article by KARTULIS (3).

may have slightly overestimated its size, for ocular micrometry is a rather inaccurate method. As to the limits of accuracy in micrometry, a competent authority says: "I assume that $0.2\ \mu$ is the limit of precision in microscopic measures, beyond which it is impossible to go with certainty."¹¹ Further, if the statement by Abbé be true (quoted from Lehmann and Neumann), that the limit of microscopic vision lies between 0.1 and $0.2\ \mu$, then, obviously, the diameter given by others for this bacillus, *i. e.*, $0.25\ \mu$, is too small.

We have further found the bacillus to be actively motile when taken from a recent culture on ascitic fluid agar and suspended in bouillon. However, it is non-motile when grown upon the ordinary media.

The ability to produce pigment and a greater adaptability to a saprophytic mode of existence suggests a closer resemblance to the organism described as *B. pseudoconjunctivitis*, but these faculties are variable ones in many species of micro-organisms. For instance, the variability in power of pigment production shown by *Staphylococcus aureus* or *B. pyocyaneus* may be mentioned. Again, it has often been noticed that micro-organisms isolated from a virulent form of a disease—*e. g.*, hemorrhagic septicemia in cattle, or cholera Asiatica—do not possess a corresponding degree of pathogenicity, and grow luxuriantly upon artificial media. So it is possible that in our case we happened to obtain a growth, not of the more virulent and less saprophytic type of organisms present in the tissues, but of those that had a tendency toward a saprophytic mode of existence.

As for the histological changes which this organism is able to produce, we cannot make any definite statements. It is possible that the marked canthosis occurring in the finger of monkey No. 261 may indicate the way in which the cauliflower-like verrucous growth of tissue occurring in the human cases was produced.

¹¹ W. A. ROGERS, *Proc. Am. Soc. Micros.*, 1883, 198.

THE PATHOLOGICAL ANATOMY OF "PARATYPHOID FEVER."*

REPORT OF A FATAL CASE, WITH BACTERIOLOGICAL FINDINGS.¹

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WITH refinements in the technic of the clinical application of the agglutination reactions, together with the more frequent application of cultural investigation of the blood in typhoid and other febrile diseases, has come the observation of a distinct set of cases of typhoid-like character that are caused by organisms intermediate between the typhoid and the colon bacillus. As these cases are clinically less severe, as a rule, than are infections with the true typhoid bacillus, there have been but few fatal results. Hence there is little known so far about the anatomic features of this disease, and its anatomic relation to true typhoid. In view of the close correspondence of the intermediate organisms and the typhoid bacillus, and the similarity of the clinical course of the cases, it might seem probable that the anatomic changes would be similar, if not identical. Up to the present time there have been reported four fatal cases with autopsy, in which the clinical course was like that of typhoid, together with the one we are about to describe; and since these correspond fairly well with one another and present quite a distinct picture, it may be considered that enough material has been collected to permit of some conclusions as to the pathologic anatomy of paratyphoid infection. If these cases represent the usual results of such infection, which would most naturally be the case, then it seems that the anatomic lesions of paratyphoid are quite different from those of true typhoid. Autopsies have so far been reported by Strong (17), Longcope (10), and Sion and Negel (15) in 1902, and Lucksch (11) in 1903. Autopsies in which the paratyphoid organism has been

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¹ Presented before the Chicago Pathological Society, October 12, 1903.

recovered have also been reported by Schmidt (13) and by Libman (9), but these were cases in which the disease was quite unlike typhoid fever, that of Schmidt being in the nature of a pyemia following an infection of the bile tracts in cholelithiasis. Libman's (9) case is described by him as resembling a cholecystitis or liver abscess, so much so that operation was performed; at autopsy healed and healing ulcers were found in the ileum. As the patient's blood reacted with the typhoid bacillus, he considers the paratyphoid infection to have been secondary to a recovering typhoid infection of the ambulatory type, mixed infections having been mentioned by other writers.

It may be necessary at this point to discuss briefly the matter of terminology to be adopted in referring to these cases. In the cases reported there has been a more or less random use of the terms "paratyphoid" and "paracolon," the former term generally applying to the clinical disease, the latter more particularly to the biologic features of the causative organism. Buxton (4) has attempted a classification harmonizing these two aspects, based upon a comparative study of the bacilli, in the following words:

"The following classification is therefore suggested:

Paracolons.—Those which do not cause typhoidal symptoms in man. A group containing many different members, but culturally alike.

Paratyphoids.—Those which cause typhoidal symptoms.

a) A distinct species culturally unlike the paracolons.

β) A distinct species culturally resembling the paracolons."

In the reported cases of infection with the members of this group of organisms the clinical features have been very variable, from those indistinguishable in most respects from typhoid, to those in which the organism has been an accidental finding in abscesses, etc., without relation to such a disease, as in the case of Widal and Nobecourt. From the clinical side, therefore, it may be well to recognize as a separate group those cases that resemble typhoid, reserving for them the term "paratyphoid," and referring to the other cases properly as "paracolon infections," recognizing that, if Buxton's classification is correct, the same organism may be responsible in each. The situation then will not be at all

different from what it now is in relation to infections with the Eberth bacillus. In this article, therefore, the term "paratyphoid" will be used as above indicated, without reference to the particular variety of organism involved.

CASE I.

The first case is that of a soldier, who died in Santa Cruz, July 31, 1900, reported by Strong (17) two years later. His sickness began July 5, with fever and rectal discharges of blood and mucus. "No departure from the ordinary type of typhoid fever." On account of unfavorable conditions existing at the time of death, the case unfortunately could not be completely studied. Autopsy was held forty-two hours after death, and there was much post-mortem change. The mesenteric lymphatics were swollen, and some, along the small intestine, were hemorrhagic. The spleen was very large and soft, and the pulp somewhat dark and considerably increased in amount. The liver showed advanced fatty degeneration. The capsule of the kidneys was not adherent; the organs were pale and yellow in color. Both the large and small intestine were normal throughout, except for a moderate catarrh and a few superficial hemorrhages. The solitary and agminated follicles showed no lesions. The other organs were normal. Stained coverslips from the liver showed a large capsulated bacillus, considered to be *B. aerogenes capsulatus*, and a smaller bacillus. Fresh smears from the spleen showed a few concentric aestivo-autumnal parasites, and a fair amount of malarial pigment. From the spleen a pure culture was obtained of an organism with the following characteristics: Bouillon was at first clouded; after a time a sediment formed at the bottom and often a pellicle at the top; hanging drop showed a motile bacillus; stained coverslips decolorized by Gram's method; in glucose agar there was a moderate gas production; in lactose agar there was no gas production; saccharose was moderately fermented; litmus milk was at first reddened after fourteen to twenty-four hours, and then turned blue after about forty-eight hours; in Smith's sugar-free bouillon there was no indol production. This organism was pathogenic for mice. No serum of the patient was saved for testing, and no agglutination tests seem to have been made. The few malarial parasites are considered by Strong insufficient to account for the illness, particularly as the patient received quinine. There was no other evident cause for the death of the patient than infection with this bacillus. A further study of the organism isolated by Strong will be found recorded by Buxton, in his article already quoted.

CASE II.

(Reported by W. T. Longcope (10), from Philadelphia.)

An Italian laborer, twenty-two years old, suffered from a sickness of about eleven days resembling typhoid. There was epistaxis, herpes labialis and nasalis, palpable spleen, and rose spots. Fever was high, and there was delirium. The Widal reaction was negative. Leucocytes, 5,900. Blood cultures made the day before death yielded a typhoid-like bacillus. Autopsy

showed a flabby heart, otherwise unchanged. Lungs congested and edematous, but no areas of consolidation. Spleen weighed 460 g.; extremely soft; surface dull, reddish gray, with a few small hemorrhages. Liver weighed 1,810 g.; large, soft, showing cloudy swelling. Intestines showed no changes beyond slight enlargement of the solitary follicles in the colon, and the presence of *ascaris lumbricoides* in large numbers. Mesenteric glands also free from changes, as were the other organs. Cultures from the heart's blood, lung, liver, and spleen gave an organism identical with the one isolated from the blood during life; no growths from the mesenteric glands, gall bladder, and cerebro-spinal fluid. The colon bacillus was also obtained from the liver.

Histologically the spleen was much congested, and the Malpighian bodies somewhat increased in size. There was no endothelioid proliferation, and no red blood-carrying cells were found. The liver showed, besides the general swelling and granulation of the cells, multiple focal necroses. In these foci cells of an epithelioid type were entirely absent, but leucocytes were present as well as fibrin. The capillaries contained few leucocytes and no thrombi. No definite lesions were found in the solitary follicles of the large intestines or in the Peyer's patches of the ileum; in one or two sections the lymphoid tissue was slightly increased, and in the germinal centers of the largest follicles one or two endothelioid cells were found. These cells occurred in no other situations in the intestinal wall. The mesenteric glands were unaltered.

CASE III.

(Reported by Sion and Negel (15), from Jassy, Roumania.)

This occurred in a man, twenty-four years old, who suffered from symptoms resembling typhoid, with cerebral manifestations, trismus, opisthotonus, paralysis of the right half of the body, and aphasia. The bacteriologic examination gave an organism corresponding with the paracolon bacillus. The anatomic findings were: Splenic swelling; parenchymatous degeneration; enteritis of a dysenteric character in the lower part of the ileum; a Laennec's vegetation in the left ventricle, with embolic foci of softening in the brain; infarcts in the spleen and kidney; bronchitis and pneumonia. As regards the intestine, it is stated that the entire intestinal mucosa was slightly reddened, while the lower part of the ileum and the ascending colon were darker red; the largest follicles were the size of millet seeds. At the lower end of the ileum the folds of mucous membrane showed gray, dirty bands, 1 mm. broad, for an extent of 10 cm., which were produced by a deposit of a bran-like material that was easily rubbed off, and under it the mucosa was less shining and deeper red. There was no swelling or ulceration of Peyer's patches whatever, nor of the solitary follicles, not even the slightest prominence or injection; the mesenteric glands were equally unaffected.

CASE IV.

At Prague during the winter of 1902-3 there was a large epidemic of typhoid, with the usual anatomic findings. There occurred one, however, in which the autopsy findings were so different from the usual picture that it seemed apparent to the examiner that he was dealing with something other than a true typhoid. Bacteriological examination showed that a paracolon

bacillus was the cause of the disease. The summary of the case, reported by Lucksch (11), is as follows:

The patient was a male, twenty-five years old, sick for a week before entrance to the hospital on December 24. The symptoms were chiefly weakness and depression; the stools became diarrhoeic, and four days before entering the hospital he had been in bed: for two days he had been unconscious. Examination showed the countenance bluish, the right conjunctiva reddened, the lips and tongue dry: the mouth would remain open. Abdomen much inflated. Spleen not palpable, but much enlarged on percussion. A few scattered rose spots on the abdomen. Bowels and bladder were emptied unconsciously: in the urine were albumen and indican. Death occurred in collapse on December 28, that is, about the twelfth day of the disease, the highest temperature recorded being on the 26th, when it reached 39° C. On the 26th the patient's blood gave, with strains of *B. typhosus*, a positive reaction in concentration of 1:40.

Autopsy was performed twenty-one hours after death. The abdomen was distended; visible mucous membranes pale; on the lower extremities punctate, large red flecks. Brain and meninges showed no changes. Diaphragm reached the fifth rib. Thyroid normal. Lungs adherent at the apices: studded over the lower lobe with small, pea-sized areas of consolidation, moist, dark red in color, and some a little raised. Pericardium normal. Heart unchanged, except pale and soft. The intestines were much distended. Liver small, soft, pale, and easily torn. No changes in the gall bladder. The spleen was slightly swollen, dark red, and soft. Kidneys pale and soft. Mucous membranes in general pale. The stomach showed a few hemorrhages in the mucosa. Small intestines much thinned (through distention), everywhere pale: neither solitary follicles nor Peyer's patches swollen. The mucosa of the large intestines was generally pale, in the cecum and ascending colon several spots where irregular transverse ulcers occurred, some with necrotic tissue shreds in the center. These occurred in groups of two or three, their margins not being swollen, and having an extent of up to 1 sq. cm. In the transverse colon the follicles, measuring 1-2 mm. in diameter, colored grayish yellow, were surrounded by a red margin 1 mm. wide. In the rectum and sigmoid flexure were no pathological alterations. The mesenteric lymph glands were generally of a dark red color, not enlarged, neither were the glands of the large intestines swollen, with the exception of one in the vicinity of the cecum, which was the size of a cherry. Pancreas and adrenals were normal.

Histology.—Liver: Slight fatty infiltration in the center of the lobules. The cellular cytoplasm was slightly granular. Spleen: Pulp elements slightly increased, but there was no demonstrable hypertrophy. Kidneys: No alteration except slight granular change in the cytoplasm. Lymph glands: In the swollen gland was an increase in lymph cells, and there were many enlarged endothelial cells with one, two, or more nuclei. No vascular congestion of the gland. Intestine: Follicles of the large intestine were not swollen, yet they contained much swollen endothelium. In the vicinity of the follicles was a slight round cell infiltration of the submucosa. The mucosa over the follicles was much infiltrated with round cells, but in places

there was still normal epithelium. Away from the follicles the submucosa showed no inflammatory reaction, and the muscularis was entirely free from it. Sections through the ulcers showed that here the mucosa and submucosa had entirely disappeared in some places, while in others there were still remains of Lieberkühn's crypts. All layers present, to the serosa, showed a moderate grade of round cell infiltration in the floor of the ulcers and for a short distance about them. There was no special swelling of the follicles in this vicinity. Blood-vessels were somewhat dilated. In places the ulceration reached to the muscularis. As the author remarks, these alterations lack the marked infiltration of the lymphatic apparatus of typhoid; they are more like those of dysentery, although not exactly the same. Bacteria were found in the ulcers, short bacilli in small numbers, that did not stain by Gram's method; but also in the superficial layers were cocci and large bacilli that retained the stain. Bacteria could not be found in the liver, spleen, kidneys, and lymph glands.

CASE V.

This case, which we desire to report, occurred in St. Luke's Hospital, Chicago, in the service of Dr. H. B. Favill, to whom we are indebted for the use of the clinical history. We also owe thanks to Dr. Gay for help in preparing the record. The patient, C. H., was a carpenter, aged forty, who entered the hospital June 9, 1903, complaining of hemorrhage from the bowels and general malaise. His sickness began two weeks before entrance with pains and stiffness in the back and legs, dull headache, and gradual loss of appetite. During this time constipation persisted for about the first six days; nine days before entrance there occurred several hard movements, not accompanied by griping. The next day he took to bed. Early on this day he vomited. He suffered no localized pain. His strength gradually decreased, and during the second week of the illness there was a hacking cough. On the morning of entrance he had a bowel movement containing much fresh blood; this was not painful or preceded by pain. He had been practically free from sickness previous to this illness, and his habits and general history were good.

Examination showed some emaciation; color pale, "muddy;" conjunctiva slightly yellowish; aspect listless, dull, but not distinctly typhoid. Circulatory system negative, except for a typical dirotic pulse; as also was the respiratory system, except for a slight hacking cough. Tongue was dry, fissured, and covered with a blackish-brown coat; protruded slowly, with tremor. The

liver was not enlarged. Spleen barely palpable at the costal margin, and slightly tender. The entire abdomen was distended, giving a tympanitic note on percussion. The skin was dry over the body, but covered with sweat over the neck and face. On the abdomen were one or two "rose spots," 2-3 mm. in diameter. Except for rachitic tibiae, the physical findings were otherwise practically negative. The urine contained from the first considerable albumin, hyalin, and granular casts, and much indican. Widal tests were made on June 9, 12, 14, and 20, all of them being negative with a dilution of 1 to 40. On June 9 the leucocyte count was 5,400; on June 12, 7,400; on June 24, 5,000. On the 24th the hemoglobin was at 70 per cent.

On the day of entrance the temperature remained at about 104° with pulse of 100 to 110, and respirations from 20 to 26. The first night, which was the fifteenth day of the disease, there was again a passage of a large amount of blood in the feces, and there were further discharges of blood during the first three days' stay in the hospital; but not later. Later the stools resembled those common in typhoid fever. The temperature was not so high during the rest of the illness, although it did occasionally reach 103°. There was occasional emesis, and on the thirtieth and thirty-third days chills occurred, followed by a rise in temperature. Death occurred on June 27, about the thirty-third day of the disease.

The subjective clinical features of this case were: (1) absence of depression; (2) absence of any active or low muttering delirium, except during the first three nights in the hospital, and the few days immediately previous to death; (3) the relatively high pulse in the last two weeks.

AUTOPSY

The autopsy was performed about seventeen hours after death. The body was that of a poorly developed, emaciated man, 151 cm. tall. Sclera white. Superficial lymph glands not enlarged. Both tibiae curved forward in the median part, with transverse flattening. Toe nails greatly deformed (onychogryphosis). There was a large bed sore over the sacrum, and a smaller one beginning over the left scapula. Subcutaneous fat was almost entirely absent.

Abdominal cavity.—Inguinal and femoral rings closed. Omentum almost devoid of fat. A few adhesions about the spleen. Appendix 5 cm. long, free from adhesions. Mesenteric lymph glands not enlarged or congested, and appear of normal consistence. The liver reaches just to the costal margin. The peritoneum is everywhere smooth and shining; there is no discoloration of the intestinal covering, except a slight reddening over the ileum just as it joins the colon. No abnormal fluid in the cavity. Retroperitoneal glands not enlarged. A few adhesions about the gall bladder, but none about the liver.

Thoracic cavity.—Both pleural cavities are free from adhesions, except a few to the pericardium, and there is no increased amount of fluid. At the site of the thymus there is a small amount of pinkish tissue. The pericardial cavity contains 30 c.c. of clear fluid; surface smooth and shining, except for a "milk spot" on the anterior surface of the right ventricle, about 5×2 cm.

Heart.—Valves, orifices, and endocardium normal in all respects. Musculature of normal consistence, but slightly pale; no increase in the connective tissue; both ventricles slightly dilated.

Lungs.—The lungs are soft and pale in the anterior portion; much darker and more firm posteriorly. Beneath the pleura are many small ecchymoses. Cut surface exudes much frothy fluid, that from the posterior part being mixed with blood. Near the posterior margin of the left lower lobe is an area 1 cm. in diameter projecting from the surface, which is quite firm, with a pale center and dark red periphery; upon cutting this open the center is white and extends 4 mm. into the lung substance, firm and flesh-like in consistence. Near the hilum of the right lung was a small puckered scar with a grain of calcareous material in the center.

Thyroid.—Not enlarged; contains little colloid.

Liver.—Of moderately firm consistence, and contains a large amount of blood. Cut surface shows lobular markings plainly; peripheries light in color.

Spleen.—Greatly enlarged, being over twice as large as normal; weight 400 g. Consistence extremely soft, and it tears very

easily. Cut surface is dark in color; Malpighian bodies difficult to discern; pulp is almost fluid in consistence.

Pancreas.—Shows no changes.

Gastro-intestinal tract.—Stomach and esophagus show no changes. In the ileum, just above the ileo-cecal valve, ulcerations are encountered. In the lowest 3 cm. of the ileum the mucosa is almost entirely ulcerated away, and the ulcerations extend upward with less abundance to a height of 8 cm. The ulcers are extremely irregular in size and shape, often coalescing, so that there are but a few separate ulcers to be distinguished. They bear no relation to the lymphatic apparatus of the bowel, but stop abruptly with the margin of the ileo-cecal valve. The floor of the ulcers does not show the muscularis in any place, although the mucosa has been ulcerated through and removed; the floor is of a dirty gray color, but there seems to be no deposition of any sort of membrane. The margins are not in the least swollen, although often slightly undermined. The bowel near the ulcers is not at all swollen or hyperemic, except a diffuse reddening just at the junction of ileum and colon. The peritoneum is not affected. The ulcerations are entirely dissimilar to those of typhoid, particularly in the lack of infiltration and in their superficiality; they resemble much more the ulcers of dysentery. Twenty-six cm. above the valve is a solitary area of swelling, with beginning ulceration, and 60 cm. above is still another. The solitary follicles, except for these areas, are not at all enlarged, and Peyer's patches are equally free from any swelling whatever—they can be found only with difficulty. As a whole, the walls of the intestine seem thin and pale. In the large intestine there is no ulceration, and no enlargement of the follicles. The mucosa of the appendix also appears normal.

Kidneys.—A little enlarged, weighing 180 g. each; consistence firm; no external changes. Cut surface dark in color, because of the presence of much blood; cortex measures 6–8 mm. in thickness, the markings being fairly distinct. The capsule strips, leaving a smooth surface. The adrenals, urinary bladder, and prostate show no changes.

HISTOLOGICAL EXAMINATION.

Intestine.—Sections taken through different ulcers show that in general the ulceration extends into the submucosa, but rarely passes through it. There seems to have occurred some post-mortem digestion of the surface. The margin of the ulcers is usually sharp, often slightly undermined, and passes abruptly through the mucosa, generally to about the middle of the submucosa. The chief feature is the absence of any evidence of reaction in the ulcerative process. The floor and margin of the ulcer is formed by tissue that has lost all affinity for nuclear stain, although retaining in part the form of the cells that originally composed it, and which stain faintly with eosin. This non-staining part fades insensibly into the tissue that appears quite normal. This absence of demarkation is similar to that seen in noma and other gangrenous ulcerative lesions. Where the margin of the ulcer passes through a lymph follicle the cells at the margin of the necrotic part are obscured by a diffuse dark blue stain, as if the nuclear substance of the lymphoid cells had diffused among them, still retaining its affinity for stain. Except for this, the presence of the lymphatic structures does not seem to have any effect on, or relation to, the ulcerative process. The portions of tissue adjacent to the ulcer seem almost entirely unaffected by the process. There may be a few more plasma cells than normal, but there is no congestion. The lymph follicles show a total absence of the endothelial proliferation of typhoid fever, phagocytes can rarely be found, and there are no thrombi except in the necrosed tissue. There are a few small hemorrhages in the submucosa, but they are rare. There is also a total absence of leucocytic invasion, and there are very few eosinophiles to be found in the tissues.

Stained by methylene blue, the tissues beneath the floor of the ulcer are found to be swarming with bacteria. On and near the surface are bacilli of various shapes and sizes, spored and non-spored, and a few cocci. Penetrating deeper into the tissue, but rarely beneath the submucosa, are bacilli of the shape and size of long colon bacilli. This bacterial growth is quite diffuse, without tendency to form colonies. Stained by Gram's method, only part

of the surface forms can be seen—few bacteria beneath the surface retain the stain—the long bacilli being quite decolorized.

Mesenteric glands.—There is a total absence of congestion or swelling of the lymph follicles and sinuses. In the sinuses, however, are numerous small areas filled with fibrin and containing few cells. They resemble focal necroses, except for the absence of any remnants of necrotic cells or cellular infiltration. The cells immediately about them are unaltered, and the fibrin passes off in threads between the cells of the normal tissue. Occasional small threads of fibrin can be found in many places in the glands. In the lymph sinuses there is some increase in the number of endothelial cells, but by no means comparable to that seen in typhoid. A few of them contain a single lymphoid cell, but phagocytosis is by no means common. There are many plasma cells in the sinuses, and a few polymorphonuclear leucocytes. The blood vessels contain many leucocytes and occasional plasma cells. The germinal centers of the follicles show no evidence of proliferation. Sections were stained by Gram's method and with methylene blue, but no bacteria could be found.

Liver.—In general the liver cells are unchanged, except that those near the center of the follicle contain a golden-brown pigment usually situated in the center of the cells. A few small but typical foci of necrosis are present, in which the destroyed liver cells have been replaced chiefly by small mononuclear cells, a few leucocytes and plasma cells, and what in some instances appear to be new-formed liver cells. Such areas are scanty, and all are small. They differ from the foci of typhoid chiefly in the absence of endothelial cells. Between the liver-cell columns are more than the usual number of polymorphonuclear leucocytes. There is no congestion or connective tissue proliferation, and bile-vessels and blood-vessels appear unchanged. In the capillaries are frequently found clumps of bacteria of the form of the colon type; they are destained by Gram's method. There is no change observable in the cells immediately in contact with these masses of bacilli, so it would seem probable that they have multiplied after death.

Spleen.—Here the changes are much like those of acute

splenic swelling of typhoid. There is the same separation of the Malpighian bodies by the congested splenic pulp, in some areas of which nothing at all can be seen but blood, as if a parenchymatous hemorrhage had occurred. In the pulp are many large endothelial cells, many of which are full of a golden-brown pigment, others containing red corpuscles in various stages of destruction, and lymphoid cells. There are many polymorphonuclear leucocytes, some of which contain pigment, and an increased number of plasmic cells. The increase in the number of cells in the pulp is so great that it seems that the increased size of the spleen is due as much to them as to the blood. No changes are seen in the Malpighian bodies, vessels and stroma. No areas of focal necrosis were found, and no bacteria could be found in sections.

Kidneys.—Except for congestion of the tufts there are no changes in the glomerules. The convoluted tubules have a low epithelium with nuclei generally small and deep-staining; the lumen is filled with granular, eosin-staining detritus. The collecting tubules are approximately normal and free from casts. The capillaries are generally distended, particularly in the medulla. No interstitial increase has occurred.

Adrenals.—No changes are found, in spite of the fact that many of the small vessels are filled with bacilli that are destained by Gram's method. The cells in the vicinity of the bacilli do not seem affected thereby. There seem to be a few more plasma and round cells in the interstitial tissue than is usual.

Lung.—The nodule found in the lung histologically presents the features of a small septic infarct. There is a central mass of leucocytes, among which are practically no remains of the lung tissue, and outside is a wide area in which the air sacs are packed with well-preserved red corpuscles. Beyond this the lung presents no features of special interest, the findings being those of hypostatic congestion and compensatory emphysema.

Myocardium.—Beyond a slight segmentation of the muscle fibers there are no changes.

Pancreas.—Except for small fibrinous thrombi in the large veins there are no changes.

Thyroid.—Presents no alterations.

Thymus.—There is quite a large amount of thymus tissue present in the lobules of fat that usually replace it, but only traces of Hassell's corpuscles remain.

Prostate.—The tubules are packed with desquamated cells, and there is a slight increase in the round cells of the muscular portion.

ANATOMIC DIAGNOSIS.

Multiple ulcerations in the mucosa of the ileum; pulmonary edema, hypostatic congestion, and compensatory emphysema; acute splenic swelling; septic infarct in the lung; chronic nephritis with congestion; decubitus; rachitic tibiae; onychogryphosis; paracolon bacillemia; focal necroses in the liver.

BACTERIOLOGICAL REPORT.

At autopsy cultures were made from the heart's blood, liver, spleen, and kidney. The heart's blood gave a mixed culture of a small bacillus and a staphylococcus. The cultures from the liver remained sterile, while those made from the spleen and kidney gave a pure culture of a short, actively motile bacillus. The latter organism has been found to belong to the group intermediate between the colon and the typhoid groups, *i. e.*, to the group of which *B. enteritidis* of Gärtner, *B. psittacosis*, the paracolon bacillus described by Gwyn, and Cushing's bacillus O. are members.

The cases of paratyphoid infection, reported by Achard and Bensaude (1), Widal (18), Gwyn (7), Cushing (6), Schottmüller (14), Brion and Kayser (3), and others, have been so fully reviewed by Buxton, and more recently by Korte (19), that it seems unnecessary to go into detail.

G. Bertnard Smith (16) has recently reported two cases of paratyphoid fever from both of which organisms belonging to the intermediate group were isolated. The organism from Case I produced gas in glucose, but not in lactose, saccharose, maltose, mannite, levulose, or dextrin. From Case II the organism produced gas in glucose, maltose, mannite, levulose, and dextrin. The organism from Case I, although undoubtedly a member of the

intermediate group, differs from the organisms isolated from all other reported cases in that it produces free gas in dextrose only. In maltose, mannite, and levulose growth occurred in the closed arm of the fermentation tube, but no free gas was produced.

Buxton (4) divides the members of the intermediate group into the paracolons, or those which do not produce typhoidal symptoms in man, and the paratyphoids—those which do produce such symptoms. This latter group is subdivided into:

Group *a*. A distinct species, differing from the paracolons in cultural characteristics. These organisms produce acid in milk, and the medium becomes alkaline in about ten days; gas is produced in yeast and glucose broths, with no increase in the amount after the first twenty-four hours; the color in neutral red agar changes completely to yellow in forty-eight hours, this color being permanent. Sera of animals immunized against members of this group give interactive agglutinations within the group, but give negative results when tested with members of Group *β*.

Group *β*. The organisms belonging to this group culturally resemble the paracolons. Permanent acidity is produced in litmus milk; gas is produced in yeast broth, the amount increasing up to the third day; a yellow color appears in neutral red agar after forty-eight hours, the original red color returning after four to five days. Agglutination tests were interactive within the group only.

BIOLOGICAL AND CULTURAL CHARACTERS OF BACILLUS FROM THE PRESENT CASE.

The organism obtained from the spleen and kidney was a short, actively motile bacillus, which was decolorized by Gram's method. The growth of the organism on agar, gelatin, broth, and potato does not differ from the growth of the typhoid bacillus.

On agar a white growth appears along the path of the needle. Gelatin is not liquefied. Peptone broth becomes clouded, but there is no pellicle formed and no sediment. Indol is not formed. Potato shows a slight growth, with no discoloration.

Milk is rendered slightly acid in from two to five days, and then changes slowly to an alkaline reaction which is pronounced

after from ten to fourteen days. A slight opalescence appears about the seventh day.

Gas production.—In testing for gas formation, fermentation tubes were used, containing sugar-free broth to which had been added 1 per cent. of the various sugars. A culture of Buxton's paratyphoid bacillus, and cultures of two paratyphoid bacilli recently studied in this laboratory by G. Bertnard Smith (16), were compared in parallel tests. The following table shows the percentages of gas formed from the various carbohydrates by these organisms:

	Dex-trose	Lac-tose	Sacch-arose	Mal-tose	Man-nite	Levu-lose	Dex-trin	Galac-tose
Bacillus from the present case.....	25	25	20	25	..	22
Buxton's paratyphoid bacillus	25	27	25	35	..	20
Smith's paratyphoid I	28
Smith's paratyphoid bacillus II.....	22	30	25	27	15	25

Neutral red agar.—After twenty-four hours the medium appeared yellow, except for a narrow band of red at the surface and at the bottom of the tube. After forty-eight hours the red band at the bottom of the tube had disappeared, but that at the surface still remained. After a lapse of ten days the tube was red, except for a slight yellow color at the bottom, the original color returning after fifteen days.

Serum reactions.—At autopsy, slides were made of the heart's blood, with a view to testing the action of the patient's serum on any organism that might be recovered. Owing to an accident, these dried-blood preparations were destroyed, and an important link in the chain of evidence concerning our paratyphoid bacillus is therefore lacking.

In making the agglutination tests there were used two cultures of *B. typhosus*, two of *B. coli*, and one culture each of *B. enteritidis*, *B. cholerae suis*, *B. paratyphosus* of Buxton, *B. icteroides* (Sanarelli), *B. dysenteriae*, and *B. fecalis alcaligenes*, together with the organism isolated from the present case. Sera from rabbits immunized against *B. coli*, *B. typhosus*, *B. dysenteriae*,

B. enteritidis, *B. paratyphosus* of Buxton, and the bacillus from this case, were tested with the various members of the colon, intermediate, and typhoid groups. In all agglutination tests an arbitrary time limit of two hours was set, in order that results obtained with the various organisms might be compared.

Serum from a rabbit immunized against *B. coli*, which agglutinated the homologous organism in dilutions of 1:400, gave no result when tested with either the organism from the present case, or with Buxton's *B. paratyphosus*. A typhoid serum of high agglutinative power, 1:12,000, gave a positive result with the paratyphoid bacillus from the case in dilutions of 1:25, but normal rabbit sera gave like results in dilutions of 1:10 and 1:15. Serum from a rabbit immune to *B. dysenteriae* agglutinated the homologous organism in dilutions of 1:5,000, but gave no result with the paratyphoid bacillus in dilutions above 1:10.

B. enteritidis serum, having an agglutinative strength of 1:7,000, gave negative results in all dilutions with both Buxton's paratyphoid bacillus and the organism from our own case.

Serum from rabbits immunized against Buxton's paratyphoid bacillus and against our own organism gave the following results: Buxton's paratyphoid serum of an agglutinative strength for its own organism of 1:40,000 gave a positive result with the paratyphoid bacillus from the case in dilutions up to 1:30,000. A dilution of 1:40,000 gave a negative result. Serum from this animal did not agglutinate the other members of the intermediate group that were tested, nor members of the colon and typhoid groups.

A serum, obtained by immunizing a rabbit against the organism from our case, agglutinated the homologous organism in dilutions up to 1:40,000, a dilution of 1:50,000 giving a negative result. Buxton's paratyphoid bacillus was agglutinated in dilutions up to 1:20,000; a dilution of 1:25,000 failed to agglutinate. *B. coli*, *B. enteritidis*, *B. cholerae suis*, *B. icteroides* (Sanarelli), *B. typhosus*, *B. dysenteriae*, and *B. fecalis alcaligenes* were not agglutinated by this serum in any dilutions. The following table shows the action of this serum upon four paratyphoid organisms.

The character of the organism isolated from the present case indicates that it is a paratyphoid bacillus belonging to group *a* of Buxton's classification.

	1:40	1:100	1:1,000	1:20,000	1:30,000	1:40,000	1:50,000
B. paratyphosus fr. the case	+	+	+	+	+	+	-
Buxton's B. para- typhosus.....	+	+	+	+	-	-	-
Smith's paraty- phoid I.....	+	+	-	-	-	-	-
Smith's paraty- phoid II.....	+	+	+	-	-	-	-

GENERAL SUMMARY.

Putting together these five cases, it is evident that paratyphoid infections are accompanied by changes quite different from those of typhoid. On the other hand, there is little characterizing this type of infection or differentiating it from other septicemias anatomically, however much it may differ clinically. The most constant change is the splenic enlargement, which was present in all five cases. In most respects this enlargement seems to be the same as that of typhoid or ordinary septicemia, and in our case the microscopic findings are also similar. The loading of the splenic endothelial cells with pigment and erythrocytes is evidently the result of the hemolysis of the disease. The intestinal lesions, however, are quite variable. Because of the occasional occurrence of intestinal hemorrhages, it had at first been thought that intestinal ulcers probably were present, although the first cases autopsied, those of Strong and Longcope, showed the intestines to be quite unaffected. In the last two cases, however, there have been numerous ulcers, although entirely different from those of typhoid. In our case, in which the hemorrhages were a prominent feature of the case, the largest amount of ulceration of any of the five was found. The reporters of the three cases in which ulcerations were described all agreed in likening the ulcers to those of dysentery rather than to those of typhoid. In all cases there was also a practical absence of any alterations of Peyer's patches or of the solitary follicles. Likewise the mesenteric glands are almost unaltered. Strong alone reports swelling of

the glands, which were not microscopically examined. Lucksch found one enlarged gland, with slight endothelial proliferation. In the other three there were no gross changes in them, and in none does there seem to have been any generalized glandular hyperplasia. In Longcope's case and in ours there were typical focal necroses in the liver, differing from those of typhoid in not containing endothelial cells. Beyond these changes there seem to be none of significance.

The anatomic changes, therefore, are simply those of a septicemia, with splenic swelling, and occasionally non-specific ulcerations in the intestine. This picture is, of course, altogether different from that of typhoid, excepting the cases of typhoid without intestinal lesions, and suggests that possibly some of the cases reported to be of this kind may have really been paratyphoid infections. The changes of proliferation and phagocytosis as described by Mallory (12) are almost entirely absent in the intestinal lesions when ulcers exist, and are very slight in the mesenteric glands, not being greater in our case than might be seen in any enteritis. The total escape of the Peyer's patches in all five cases suggests some essential, if obscure, biologic difference between the typhoid and paratyphoid organisms.

The slight alterations generally found in the intestines agrees with the clinical history in respect to intestinal symptoms, for Brion (2) found diarrhoea present in but 18 per cent. of the recorded cases. Hemorrhage was present in but 5 per cent. Hence it is quite probable that intestinal lesions may be even less frequent in the non-fatal cases than indicated in the five autopsies, since these last necessarily represent exceptionally severe types of the disease. That fatal paratyphoid cases are really rarities, rather than overlooked through imperfect bacteriologic study, is indicated by Lucksch's (11) experience, for his one paratyphoid case was the only one found among 102 autopsies of typhoid cases. Another interesting feature is the scattering of these five cases over the entire globe, occurring as they did in Santa Cruz, Bohemia, Roumania, Philadelphia, and Chicago, which indicates that the geographical distribution of the paratyphoid bacillus is as wide as that of the typhoid bacillus.

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EXPLANATION OF PLATE III.

FIG. 1.—Low-power drawing of the margin of one of the intestinal ulcers. The blue-staining area in the submucosa is the edge of a Peyer's patch, at the line of junction of the necrotic and sound tissue, showing the diffusion of the nuclear material as described in the text. With this exception, it will be noticed, there is a total absence of any line of demarkation, as well as of cellular reaction. The absence of proliferation is particularly striking if compared with Mallory's (12) plates.

FIG. 2.—High-power drawing of mesenteric lymph gland, showing one of the areas resembling focal necrosis. The pink-staining substance is fibrin. The absence of necrotic cells and proliferated endothelium is shown.

We are indebted to Miss E. P. Miller for the drawings.

AGGLUTINATION OF STREPTOCOCCI, ESPECIALLY THOSE CULTIVATED FROM CASES OF SCARLA- TINA, BY HUMAN SERA.*

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EVER since it has been known that immune sera often agglutinate the bacteria concerned in their production, constant efforts have been made by investigators to determine the value of this reaction in the case of various bacteria, both as a means of diagnosis of specific diseases and as a method of differentiating bacteria which closely resemble each other. Streptococci have been included among the bacteria thus studied, and certain facts have been learned regarding the power of sera to cause their agglutination.

The first demonstration of the agglutination of streptococci by immune sera was furnished by van de Velde.¹ He studied the action of the sera of horses which were artificially immunized against streptococci, and found that an antistreptococcus serum possessed rather weak agglutinating power over the homologous bacterium, but not over other strains of streptococci. In 1897 Bordet² also determined that antistreptococcic serum possesses agglutinating properties, though in a feeble degree. He found that the agglutination of streptococci by preventive serum is usually distinct, but never great, and that a considerable quantity of serum is required to bring it about, at least one-third the volume of a fluid culture. R. Kraus,³ writing upon agglutination, refers to observations by Widal in man which gave uncertain and variable results. His own investigations in this direction had not been satisfactory, and he concluded that little is to be expected from serum-diagnosis in streptococcus infections.

In 1902 Aronson⁴ made very complete observations upon the agglutinating action of the sera of horses which had been immu-

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¹ *Arch. de Méd. expér.*, 1897, 9, 835.

² *Ann. de l'Inst. Pasteur*, 1897, 11, 177.

³ *Wien. klin. Wchnschr.*, 1899, 12, 95.

⁴ *Berl. klin. Wchnschr.*, 1902, 39, 979-982; 1006-1010.

nized against streptococci. The streptococci employed in the immunization and in the tests were rendered highly virulent by multiple passage through mice. He found that the serum from a horse treated with a certain streptococcus agglutinated other cultures of streptococci as well as the homologous one. His observations point to a close relationship between all streptococci. Agglutination was observed in dilutions of thirty times or less. There was no correspondence between agglutination and protective properties, and agglutination did not interfere with the life of the cocci.

Shortly after Aronson's publication, Moser and Pirquet⁵ reported observations in connection with streptococci, and especially those coming from cases of scarlatina. Their findings were in some particulars exactly opposite to those of Aronson. From their experiments they arrived at the conclusion that streptococci from cases of scarlatina are different from those found in other human diseases. They tested the agglutinating power of human sera upon a streptococcus cultivated from the heart's blood of a fatal case of scarlatina in a child, with the following results:

1. Serum from cases of scarlatina agglutinates in slight dilutions in one-half the cases, the maximum dilution being 1:8.

2. Serum of other children agglutinates but rarely (three out of twenty-eight examinations), the maximum dilution being 1:4.

3. High agglutinating sera of horses injected subcutaneously in human patients always confers agglutinating properties upon human serum which are generally proportional to the quantity of serum injected and reach their highest point after twenty-four to thirty-six hours.

They found that normal horse serum agglutinated streptococci from various sources, but in moderate dilutions only, 1:4 to 1:64. The serum of horses immunized with streptococci cultivated from the heart's blood of fatal cases of scarlatina, and not passed through animals, agglutinated the same streptococci in very considerable dilutions (1:1,000 to 1:64,000). The same effects were produced upon streptococci cultivated from the heart's blood of cases of scarlatina, which were not used in

⁵ *Wien. klin. Wchnschr.*, 1902, 15, 1086.

producing the immunity (1:1,000 to 1:16,000). Streptococci from other diseases were agglutinated by the same sera at only a little higher dilutions than by normal horse serum (1:4 to 1:250), and the sera of horses immunized with these latter streptococci agglutinated streptococci from cases of scarlatina only in the same proportion as normal horse serum.

Fritz Meyer⁶ investigated the agglutination of streptococci by various antistreptococcic sera. He found that streptococci are agglutinated by their corresponding immune sera. By this means he undertakes absolutely to differentiate streptococci of anginas (scarlatinal, rheumatic, simple) from those of pyogenic infection. He finds gradual differences in the reaction of the anginal streptococci which speak against an identity among them.

Wlassjewski⁷ studied the agglutinating effects of antistreptococcic serum (polyvalent), and of human serum from cases of puerperal fever, rheumatism and from healthy persons. The streptococci employed were from cases of scarlatina, puerperal fever, erysipelas, phlegmon, and dysentery. He found the polyvalent antistreptococcus serum agglutinated all the streptococci employed in the immunization, but some more strongly than others. The serum from puerperal cases agglutinated the puerperal streptococci at a dilution of 1:400 after twenty-four hours at 37° C. Serum from a case of rheumatism had no effect upon any of the cultures. He concludes that the reaction of agglutination is apparently specific, and may become a useful means of diagnosis and furnish a rational indication for the use of antistreptococcus serum in certain cases.

The agglutinating action of serum from cases of scarlatina upon scarlatinal streptococci has been studied also by Salge.⁸ He found that streptococci from scarlatina were agglutinated by scarlatinal sera in dilutions up to 1:500. Agglutination did not occur with streptococci from other sources, nor with normal sera. Sera from other streptococcus diseases were not tested. He arrived at the conclusion that a distinct biologic relationship exists between scarlatinal streptococci and the serum of scarlatinal patients.

⁶ *Deutsche med. Wchnschr.*, 1902, 28, 751.

⁷ *Centralb. f. Bakteriöl.*, I. Abt., "Referate," 1903, 33, 464.

⁸ *Ibid.*, 32, 643.

Stimulated by the reports of Moser and Pirquet, and Meyer, which appeared to indicate that varieties of streptococci could be recognized by differences in the agglutinating effects of anti-streptococcus sera upon them, Aronson⁹ undertook another series of exhaustive studies in order to learn whether his former results were reliable or not. In order that the conditions might correspond with those in the experiments of Moser and Meyer, horses were immunized with cultures of streptococci cultivated directly from man without passage through animals. The following cultures were employed for separate horses: seven various scarlatinal cultures, scarlatinal culture No. 8, puerperal sepsis No. 1, puerperal sepsis No. 2, articular rheumatism No. 1, articular rheumatism No. 2, otitis. Many other streptococci besides those employed in immunizing the horses were tested as to the agglutinating power of the various horses' sera upon them. Aronson found that streptococci cultivated directly from man are more readily agglutinated than those rendered virulent by animal passage. He also observed that cultures which inclined to produce a conglomerate growth were more easily agglutinated than those growing diffusely. The conclusion is reached from his agglutination tests that different groups of streptococci cannot be separated by this means. In his former work he had found that a serum prepared by immunizing with streptococci made virulent for mice agglutinated all such virulent cultures alike, but he now found that the serum prepared by immunizing with streptococci cultivated directly from man agglutinated in an entirely different way. The reaction with the latter serum was not obtained with all streptococci, nor with single groups, but was an essentially individual reaction. It was entirely indifferent whether the test culture originated from the same human disease or not. Not infrequently a readily agglutinated culture was agglutinated more by a serum prepared with a septic streptococcus than by one prepared with a scarlatinal streptococcus. The serum from the horse immunized with seven scarlatinal cultures agglutinated all the cultures employed. It agglutinated one scarlatinal streptococcus at a dilution of 1:20,000, another at 1:20, and a third not at all.

⁹ *Deutsche med. Wochenschr.*, 1903, 29, 439.

The serum from the horse immunized with scarlatinal culture No. 8 agglutinated the culture employed at 1:4,000, another scarlatinal culture at 1:50, while the serum prepared with a septic streptococcus agglutinated the latter at 1:100. The septic streptococcus No. 1 was clumped by its homologous serum at 1:3,000, and not at all by the serum produced by septic streptococcus No. 2. Of the sera prepared from the two streptococci from articular rheumatism, each agglutinated its homologous culture, but not the other. Aronson concludes that in no case can a differentiation be made between streptococcus groups by the most careful agglutination tests.¹⁰

Early in the year 1903 the investigations about to be recorded were undertaken to determine whether streptococci from cases of scarlatina were constantly agglutinated by the sera of scarlatinal patients, and, if so, whether this was specific and might be of value in the diagnosis of doubtful cases. The technic employed has not differed materially from that of others who have used the macroscopic test. Some preliminary tests were undertaken by the microscopic method, but the results were usually so uncertain that it was abandoned. Aronson has insisted upon the necessity of using the macroscopic method for all non-motile bacteria in agglutination tests, and our results confirm his statements.

The cultures used were made in bouillon which was neutral to litmus and 1 per cent. acid to phenolphthalein. The growth in such bouillon was usually uniform. Each lot of bouillon before being used was tested with a culture known to be readily agglutinated, and with a serum previously found to agglutinate the culture; and if agglutination was not obtained, the entire lot of

¹⁰ Since this article went to the printer, two noteworthy communications bearing upon this subject have appeared. NEFFELD (*Zeit. f. Hyg. u. Infec.*, 1903, 44, 161) has studied immunity and agglutination in connection with streptococci. He found, like Aronson, that avirulent streptococci are more readily agglutinated than virulent ones. He also concludes that no specificity of streptococci isolated from scarlatina has been shown. MOSER AND PIRQUET (*Centralbl. f. Bakteriöl.*, I. Abt., "Originale," 1903, 24, 560 and 714) have elaborated the microscopic method of studying agglutination of streptococci, and find it as reliable as the macroscopic. Their results do not show that the sera of horses immunized against streptococci from scarlatina produce agglutination of all streptococci cultivated from the blood of cases of scarlatina. They found the blood serum from cases of scarlatina to agglutinate a streptococcus from the blood of scarlatina, but only at low dilutions, not greater than occurred with sera from other diseases. Little value attaches to these observations, since only a single culture was employed, and the character of the control cases is not stated in detail.

medium was discarded. Sometimes the reaction of the bouillon appeared to be at fault, a slight deviation in either direction being sufficient to interfere with the agglutination. If a culture in bouillon was found to be distinctly acid to litmus, it was not agglutinated by a serum which agglutinated the same streptococcus when grown in bouillon which remained neutral after twenty-four hours. If bouillon contained a small amount of sugar, the result-

TABLE I.

Showing that a Culture in Glucose Bouillon Becomes Agglutinable when the Acid in It is Neutralized.

Serum from Case of Scarlatina, No. 289	Strepto- coccus Culture No. 37, Grown in Plain Bouillon	Streptococ- cus Culture No. 37, Grown in 0.25 per Cent. Glucose Bouillon	$\frac{n}{20}$ Solution NaHO	Agglutina- tion after Twenty- Four Hours at Room Tempera- ture
.....	1	0
0.004	1	+
.....	..	1	0
0.004	..	1	0
0.004	..	1	0.033	0
0.004	..	1	0.066	+
0.004	..	1	0.132	+
0.004	..	1	0.198	0
0.004	..	1	0.264	0
.....	..	1	0.033	0
.....	..	1	0.066	0
.....	..	1	0.132	0
.....	..	1	0.198	0
.....	..	1	0.264	0

ing production of acid interfered with agglutination. If alkali was added to such a culture in varying quantities, agglutination would occur near the neutral point (Table I). The effects of acid and alkali upon agglutination are shown in the accompanying tables (II and III). Cultures which grew conglomerately were not employed. It was found that cultures which at first grew conglomerately lost this property if grown for several generations in glucose bouillon with daily transplantations. Cultures which at first grew conglomerately were better agglutinated than those growing diffusely, as pointed out by Aronson.

The serum was obtained from blood drawn from one of the veins at the elbow by means of a Luer syringe, all precautions to

TABLE II.

Showing the Effects upon the Agglutination of the Additions of Alkali and Acid.

Serum from Scarlatina Case 292	Strepto- coccus Culture No. 37, Grown in Plain Bouillon	$\frac{n}{20}$ Solution NaHO	$\frac{n}{20}$ Solution H ₂ SO ₄	Agglutina- tion after Twenty- Four Hours at Room Tempera- ture
0.008	1	0.033	0
0.008	1	0.016	0
0.008	1	0.008	0
0.008	1	0.004	0
0.008	1	0.002	+
0.008	1	0.001	+
0.008	1	+
0.008	1	0.001	+
0.008	1	0.002	+
0.008	1	0.004	+
0.008	1	0.008	+
0.008	1	0.016	+
0.008	1	0.033	+
0.008	1	0.066	0
0.008	1	0.132	0

TABLE III.

Showing the Effects upon the Agglutination of a Streptococcus by a Normal Rabbit's Serum, Produced by the Addition of Alkali and Acid.

Normal Rabbit's Serum	Strepto- coccus Culture No. 45, Grown in Plain Bouillon	$\frac{n}{20}$ Solution NaHO	$\frac{n}{20}$ Solution Lactic Acid	Agglutina- tion after Twenty- Four Hours at Room Tempera- ture
0.125	1	0.125	0
0.125	1	0.062	0
0.125	1	0.031	0
0.125	1	0.015	0
0.125	1	0.007	+
0.125	1	0.0035	+
0.125	1	+
0.125	1	0.0035	+
0.125	1	0.007	+
0.125	1	0.015	+
0.125	1	0.031	+
0.125	1	0.062	+
0.125	1	0.125	+
0.125	1	0.25	0
0.125	1	0.50	0

avoid contamination being observed. The serum was allowed to separate from the clot in the ice-box, and when used, a culture was made from the serum to insure its sterility. In making a series of tests, the serum was diluted with the same bouillon as that used for the culture. The dilutions of serum were of strengths of $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, etc. One part of these various strengths of serum was added to 30 parts of the twenty-four hours' bouillon culture. The mixtures of 1 part of serum to 8 parts of culture were made by means of drops from pipettes of uniform size. The mixtures of serum and culture were made in small test-tubes, each containing about 1 c.c. These were kept at room temperature for twenty to twenty-four hours, and then examined. When agglutination had taken place, the bacteria were collected at the bottom of the tube, leaving the upper part clear, and on shaking they rose in the fluid in flakes and granules which were not broken up by considerable agitation. When there was no agglutination, the bacteria which had settled to the bottom were uniformly distributed throughout the fluid on agitation. A control of the culture without any serum added to it was always kept in the same-sized tube and in the same quantity as the test mixtures; and if this control showed any conglomeration, the entire series was discarded. Microscopic examination of the clumps in the tubes exhibiting agglutination showed them to be made up of closely packed cocci. The chain arrangement was scarcely at all perceptible in these bunches. The rapidity with which agglutination appears is variable. It occurs more rapidly at incubator than at room temperature, often being complete in one hour, and usually in three to five hours. It was found that a corresponding agglutination took place after eighteen to twenty-four hours at room temperature, and this time was followed in our experiments. Room temperature has the advantage that little growth of streptococci occurs in twenty-four hours. If the mixtures are to be placed in the incubator, it would seem desirable to add formalin to the culture. We have found that 1 per cent. formalin has no effect upon the agglutination. In part of the sera tested the highest dilution of serum which produced agglutination was determined. In others the dilutions were carried to

some point below the limit, lack of serum preventing further determinations.

In the tests here recorded the following cultures of streptococci were employed:

1. Eleven from cases of scarlatina: Nos. 11, 27, 28, and 36 from the tonsil, Nos. 37 and 47 from the blood during life, No. 39 from the lung after death, Nos. 40, 45, and 46 from the blood of heart after death, No. 44 from the pericardial exudate after death.
2. Two from phlegmons, Nos. 29 and 48.
3. One from the blood of a case of puerperal fever during life, No. 31.
4. One from the heart's valve in ulcerative endocarditis.
5. One from the peritoneal exudate in perforative peritonitis.
6. One from strangles in horses.
7. One from a rabbit after multiple passages.

Upon some of these cultures the agglutinating powers of each of the human sera from the following sources were tested:

a) Twenty-one cases of scarlatina. These represented all degrees of severity, and the blood was drawn from the second to the eighteenth day. Thirty specimens of sera were tested, twenty being obtained during the first week of the disease.

b) Seventeen cases of lobar pneumonia (nineteen sera). For these sera I am much indebted to Dr. E. C. Rosenow.

c) Four cases of erysipelas.

d) One case of endocarditis.

e) Three cases of measles.

f) Six cases of puerperal fever.

g) Two cases of typhoid fever.

h) Four normal individuals.

The accompanying table (IV) shows the agglutinating action observed with the sera from cases of scarlatina upon streptococci from cases of scarlatina. Cultures 28, 37, 45, 46, and 47 were agglutinated by each serum with which they were tested, the limit of dilutions at which the agglutination occurred varying from 1:30 to 1:4,000. Cultures 27, 39, 40, and 44 were not agglutinated by any serum with which they were tested. The sera which had no effect upon these latter cultures were always tested upon Nos. 28 and 37, and found to be active against them. It was not possible to demonstrate any connection between the dilution at which agglutination occurred, and the type of the case or the time in the disease when the serum was drawn.

The agglutinating effects of sera from lobar pneumonia upon cultures of streptococci from cases of scarlatina is shown in the accompanying table (V). It will be observed that the aggluti-

TABLE IV

Showing the Agglutination of Streptococci from Cases of Scarlatina by the Sera from Cases of Scarlatina

Sera from Cases of Scarlatina		Streptococci from Cases of Scarlatina										
		11	27	28	36	37	39	40	44	45	46	47
		Tonsil	Tonsil	Tonsil	Tonsil	Blood During Life, Case 270	Lung, Post-Mortem, Case 285	Blood, Post-Mortem, Case 285	Pericardial Exudate, Post-Mortem, Case 298	Blood, Post-Mortem, Case 300	Blood, Post-Mortem, Case 299	Blood During Life, Case 312
Number of sera tested (cases).....		6(5)	2(2)	22(16)	3(3)	22(16)	5(4)	3(3)	2(2)	2(2)	5(5)	2(2)
Number of sera with which ag- glutination was ob- tained at various di- lutions	1:8	2	3	2	4	..
	1:30	1	3
	1:60	1	..	3	..	1	1	1
	1:120	2	..	8	1	8
	1:240	6	..	3	1
	1:480	4	..	2
	1:1000	3
	1:2000	1
1:4000	1	..	1	
Number of sera with which the limit of ag- glutination was deter- mined at various di- lutions	1:8	2	1	..
	1:30	1
	1:60	1	..	2	..	1	1	1
	1:120	1	1	3
	1:240	3	1
	1:480	4	..	2
	1:1000	2
	1:2000	1
1:4000	1	..	1	
Number of sera with which no ag- glutina- tion was obtained		1	2	0	1	0	5	3	2	0	0	0

nating action of the pneumonic sera was but little less than that of scarlatinal serum upon the same cultures.

Erysipelas serum possesses considerable agglutinating power over streptococci cultivated from cases of scarlatina, as shown in

Table VI. One erysipelas serum agglutinated streptococcus culture 37 at a dilution of 1:12,000, which was higher than was

TABLE V.

Showing the Agglutination of Streptococci from Cases of Scarlatina by Sera from Cases of Lobar Pneumonia.

<i>Sera from Cases of Lobar Pneumonia</i>		Streptococci from Cases of Scarlatina						
		11	27	28	36	37	39	40
		Tonsil	Tonsil	Tonsil	Tonsil	Blood During Life, Case 270	Lung, Post-Mortem, Case 285	Blood, Post-Mortem, Case 285
Number of sera tested (cases)		9(7)	3(3)	18(16)	1(1)	4(4)	1(1)	2(2)
<i>Number of sera with which agglutination was obtained at various dilutions.</i>	1:8	3	..	4	1
	1:30	2
	1:60
	1:120	3	..	4	..	3
	1:240	1
	1:480	1
	1:1000	2
	1:2000	1
<i>Number of sera with which the limit of agglutination was determined at various dilutions.</i>	1:8	1	..	3	1
	1:30	2
	1:60
	1:120
	1:240
	1:480	1
	1:1000
	1:2000
<i>Number of sera with which no agglutination could be obtained.</i>		3	3	4	0	0	1	2

ever obtained with any other serum tested. The case from which this serum came was very severe and terminated fatally. The limit of dilution at which the sera from cases of puerperal fever will agglutinate these streptococci was not determined in most cases. The dilutions are, however, sufficiently high to indicate that agglutinating power is present in considerable degree (Table

VII). Sera from two cases of typhoid fever were tested upon scarlatinal streptococci. Both sera agglutinated Culture 47 at dilutions of 1:1,000, and Cultures 28, 37, 45, and 46 at dilutions of 1:30 to 1:120. Sera from three healthy persons were found

TABLE VI.

Sera Showing the Agglutination of Streptococci from Scarlatina by Sera from Cases of Erysipelas.

Sera from Four Cases of <i>Erysipelas</i>		Streptococci from Cases of Scarlatina						
		11	27	28	36	37	39	40
		Tonsil	Tonsil	Tonsil	Tonsil	Blood, Dur- ing Life, Case 270	Lung, Post- Mortem, Case 285	Blood, Post- Mortem, Case 285
Number of sera tested.....		1	1	4	1	3	2	1
Number of sera with which ag- glutination was obtained at vari- ous dilutions.	1:8	1
	1:30
	1:120	1	..	1
	1:2000	2	..	1
	1:12000	1
Number of sera with which the limit of agglu- tination was de- termined at vari- ous dilutions.	1:8	1
	1:30
	1:2000	2	..	1
	1:12000	1
Number of sera with which no agglutination could be ob- tained.		0	1	1	1	0	2	1

to agglutinate part of the scarlatinal cultures at 1:4 to 1:15, but never higher. The sera from measles and a case of endocarditis had but feeble agglutinating power over this group of streptococci.

The streptococci from other sources than scarlatina were tested in a similar manner, and the results may be briefly summarized as follows: Culture 31, obtained during life from the blood of a woman with puerperal fever, was agglutinated by two

scarlatinal sera at 1:30, by five at 1:8, and by two not at all; by one pneumonic serum at 1:120, by one at 1:30, by four at 1:8, and by eight not at all. Of the four erysipelas sera, one agglutinated it at 1:30, two at 1:8, and one not at all. Cultures 30

TABLE VII.

Showing the Agglutination of Streptococci from Cases of Scarlatina by the Sera from Cases of Puerperal Fever.

Sera from Six Cases of Puerperal Fever		Streptococci from Cases of Scarlatina						
		11	28	36	37	44	45	46
		Tonsil	Tonsil	Tonsil	Blood During Life, Case 270	Pericardial Exudate, Post-Mortem, Case 298	Blood, Post-Mortem, Case 300	Blood, Post-Mortem, Case 309
Number of sera tested		1	4	1	5	2	4	2
Number of sera with which agglutination was obtained at various dilutions.	1:8	1
	1:30	1	1	..	1	..	2	..
	1:60	1	..
	1:120	..	1	1	1	..	1	..
	1:240	1
Number of sera with which the limit of agglutination was determined at various dilutions.	1:8	1
	1:30
	1:60	1	..
	1:120	1	..
Number of sera with which no agglutination could be obtained.		0	2	0	2	2	1	0

and 48, obtained from the pus of phlegmous, were agglutinated by various sera in a dilution of 1:8, but not higher. The other streptococci mentioned in the list were not agglutinated at all by most sera, and only with slight dilutions by a few.

Streptococci which had been agglutinated by scarlatinal and erysipelas sera were found to be alive after twenty-four hours. It does not follow from this, however, that agglutination is dependent upon any vital action of the streptococci. Cultures

Two of the sera lost their power of bringing about agglutination after being subjected to a temperature of 60–62° C. for one hour. One of the sera was still able to agglutinate one of the cultures, but in a reduced degree. These experiments show that streptococci possess a labile, functional substance which is readily destroyed by heat, as has been shown to be the case in typhoid bacilli. A second, more resisting substance, such as has been found in typhoid bacilli by Joos,¹⁰ does not appear to be present. They also show that the substances in agglutinating serum which are essential for the production of visible agglutination are readily destroyed by heat. Posselt and Sagasser¹¹ have shown by specific absorption experiments that in the process of immunization with typhoid, coli, dysentery, and cholera bacteria the *Hauptagglutinin* is not only increased as the immunization progresses, but that also the *Nebenagglutinine* are increased; and while the dilution at which the injected organism is agglutinated is increased, there is also an increased agglutinating action upon the related bacteria. The tests upon the agglutinating effect of typhoid serum upon streptococci which have been referred to, while few in number, seem to indicate that in a similar manner substances which agglutinate streptococci are formed during typhoid fever. This is the more surprising as there is no intimate biologic relationship between the organisms concerned.

The results obtained by Aronson when he tested the agglutinating properties of immune sera produced by injecting streptococci grown directly from man correspond very closely to the results here recorded where human sera have been employed.

In looking for an explanation for the different results obtained by various observers, several factors suggest themselves as perhaps able to account for them in part at least. In Salge's experiments¹² conclusions are drawn from the results of tests with scarlatinal serum only, and without the essential controls. Aronson has pointed out that trikresol soon destroys the agglutinating properties of antistreptococcic sera, and so the value of many comparisons of the relative agglutinating power of different anti-

¹⁰ *Centralbl. f. Bakteriöl.*, I Abt., "Originale," 1903, 33, 762.

¹¹ *Wien. klin. Wchnschr.*, 1903, 17, 691.

¹² *Loc. cit.*

streptococcic sera is destroyed. As has been pointed out, slight variations in the composition and reaction of the culture medium may exert a marked effect upon the reaction.

CONCLUSIONS.

1. Of streptococci cultivated from cases of scarlatina, some are agglutinated by almost all scarlatinal sera, but at dilutions varying from 1:60 to 1:4,000; others are agglutinated by the same sera with less constancy and at lower dilutions, and many are not agglutinated at all.

2. Streptococci cultivated from cases of scarlatina are agglutinated by sera from cases of lobar pneumonia and erysipelas at about the same dilutions as by scarlatinal sera, and in the case of erysipelas at even higher dilutions.

3. The same appears to be true of typhoid-fever serum, so far as limited tests indicate, and to almost the same extent of puerperal-fever serum.

4. The agglutination reaction between the streptococci cultivated from cases of scarlatina and the serum from cases of scarlet fever is in no way specific, and cannot be of any value as a means of diagnosis.

5. The effects here shown to be produced upon the agglutinating and agglutinable substances by heat, and the marked variations dependent upon slight alterations of reaction, serve to emphasize the importance of very exact methods in the study of agglutination phenomena in connection with streptococci, as well as with bacteria in general.

THE EFFECTS ON STREPTOCOCCI OF SERA OF COLD-BLOODED ANIMALS.*

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THE bacteriological investigations of scarlet fever have shown that streptococci practically always are associated with many of the severe complications of this disease, and that these organisms can be isolated from the internal organs and bone marrow of a large percentage of the fatal cases. These organisms can also be isolated from the blood, during life, in a small percentage of cases, as was shown by Hektoen, Lemoine, Slawyk, and others. Although it cannot be claimed that these investigations have shown that scarlatina is a streptococcus disease, there can be no doubt but that the complications and mortality of this disease would be greatly reduced if we were able to cope successfully with the secondary streptococcus infections.

Exhaustive studies of streptococcus infections are especially indicated in view of the fact that at present but little is definitely known about the finer mechanisms of these infections. Normal human serum is strongly bactericidal, *in vitro*, for some pathogenic bacteria, as *B. typhosus* and *B. dysenteriae*. The all-important question here is: What brings about the conditions which permit these organisms to multiply in the body and produce their respective disease? Streptococci, on the other hand, multiply very rapidly in normal human serum and in scarlatinal serum, as was shown by Dr. Weaver and myself.¹ This would seem to indicate that the body finds no protection against streptococci in the serum alone, but that the struggle between the body and these organisms must be looked for elsewhere than in the blood serum. Denys and LeClef,² Bordet,³ and Marchand⁴ have

* Received for publication October 15, 1903.

¹ *Trans. Chicago Path. Soc.*, 1903, 5, 285; *Medicine*, July, 1903.

² *La Cellule*, 1895, 11, 177.

³ *Ann. de l'Institut Pasteur*, 1897, 11, 177.

⁴ *Arch. de Méd. expér.*, 1895, 10, 253.

endeavored to show that this struggle falls entirely upon the phagocytes—a view that has not been very widely accepted, although it has many points in its favor. In other diseases, as for instance, diphtheria, antitoxins can be demonstrated in the blood serum of convalescents, but according to Neufeld,⁵ no such antibodies can be detected in the serum of convalescents from streptococcus infections. And the investigations of Dr. Weaver appear to show that as yet but little faith can be placed upon the significance of the agglutinative reactions of streptococci with the serum of scarlatinal and other patients.

One of the most interesting developments in the recent study of streptococci is the discovery by Besredka of streptocolysin, a special hemolysin produced by virulent streptococci when grown in heated rabbit serum. I have shown that this lysin is composed of a haptophore and a toxophore group, which are firmly linked together.⁶ The haptophore group may be neutralized with chicken serum, and the toxophore group can be destroyed with traces of zinc chloride or zinc sulphate. The filtrates of virulent streptococci in heated serum were found to be toxic for rabbits. The organs (especially the spleen) of a rabbit which has died from the effects of an injection with these filtrates give a strong reaction for hemosiderin, which would indicate that hemolysis has taken place during life. These results suggest that further insight into the pathogenic action of streptococci may be obtained by the minute study of the substances they produce.

Since human serum and the sera of most warm-blooded animals are not bacteriolytic for streptococci, it would be interesting to determine whether there are any sera among the cold-blooded animals which are destructive for these organisms. This work was undertaken at Wood's Hole with that object in view, but also bearing in mind the possibility of producing in some of these animals an anti-streptococcic serum of greater therapeutic value than are those so far obtained from the horse.

The sera of twenty species were tested on several strains of streptococci, by the usual plate method. The blood was obtained directly from the heart by means of a sterile pipette, allowed to

⁶ *J. Am. Med. Ass.*, 1903, 41, 962.

⁵ *Deutsche med. Wchnschr.*, 1897, 23, 162.

clot, and the clear serum which exuded was used within three to five hours after killing the animal. One c.c. of serum was introduced into each of several small tubes, inoculated with streptococci, and glucose agar plates made at intervals. The tubes were kept in the incubator at 35° C. The counts of the colonies, which were made in about thirty hours, showed that

TABLE I.

The Growth of Streptococci in the Sera of Various Cold-Blooded Animals.

Strepto- cocci	Sera	Immediately	Two Hours	Five Hours	Twenty-Four Hours
270	Butterfish	500	1,500	10,000	30,000
244	Dogfish	2,400	2,500	3,500	2,500
270	Conger eel	2,500	5,000	10,000
244	Flounder	100	500	1,900	5,000
270	Mackerel	1,300	1,300	5,000	∞ ¹
244	Butterfly ray	4,200	5,000	∞
B104	Sting ray	5,000	5,000	∞
244	Sand shark	1,400	6,000	10,000
270	Dusky shark	150	2,000	6,000
244	Scup	600	1,200	800	8,000
244	Common skate	2,000	2,400	4,500	10,000
270	Squetrague	1,000	2,000	8,000	∞
270	Common squid	200	1,000	∞
270	Sturgeon	400	900	4,000	15,000
270	Lobster	3,000	5,000	10,000	3,000
244	King crab	13,000	19,000	800
244	Spider crab	900	10,000	∞

Sources of streptococci: No. 244 from blood, during life, in puerperal sepsis; No. 270 from blood during life, in scarlet fever; No. B104 from an abscess in a guinea pig which had been injected with a fungus.

the streptococci multiplied rapidly in most of the sera, as is shown by Table I.

The sera of several species of turtles, however, showed a very different behavior toward the streptococci than did the sera of the other animals. In these sera the organisms did not multiply, but their number decreased very markedly during the first five hours, as shown by Table II. In no instance was I able to get entirely sterile plates in twenty-four hours, although in several experiments the number of colonies dropped from several thousand to less than one hundred. Control tubes were always made, and the

¹ 8 stands for very many.

results noted only when the controls remained sterile. There was one strain of streptococci (No. 244) which multiplied in all sera, although less rapidly in those of turtles than in those of the other animals. If the serum was heated to 54° C.,

TABLE II.
The Growth of Streptococci in the Sera of Turtles.

Strepto- cocci	Sera	Immediately	Five Hours	Twenty-Four Hours
B104	Speckled turtle	2,400	450	1,000
270	Speckled turtle	2,700	900	1,500
244	Speckled turtle	2,500	3,000	4,000
B104	Speckled turtle (heated)	1,800	15,000	∞
300	Painted turtle	3,400	480	1,800
B104	Painted turtle	2,000	400	1,200
244	Painted turtle	6,000	10,000	10,000
300	Painted turtle (heated)	2,500	∞	∞
B104	Yellow-bellied terrapin	9,000	3,000	480
270	Yellow-bellied terrapin	5,000	1,900	560
244	Yellow-bellied terrapin	2,300	4,500	2,800
300	Yellow-bellied terrapin	13,000	2,800	475
300	Yellow bellied ter. (heated)	10,000	60,000	∞
300	Heated serum +0.2 normal turtle serum	1,600	1,100	600
300	Bouillon +0.2 normal turtle serum	1,700	850	8,000
300	Human serum	3,300	25,000	∞
300	Human serum +0.2 turtle serum	2,100	1,600	20,000
300	Heated human serum +0.2 turtle serum	1,800	1,000	8,000

Sources of streptococci: No. 244 from blood, during life, in puerperal sepsis; No. 270 from blood, during life, in scarlet fever; No. 300 from heart's blood of scarlet-fever patient, post-mortem; No. B104 from an abscess in a guinea pig which had been injected with a fungus.

there was a rapid multiplication of all the organisms from the start. Heated serum can be reactivated by adding 0.2 c.c. of normal serum to 1 c.c. of heated serum. This does not appear to be a true reactivation, however, because nearly the same effect can be obtained by using bouillon instead of heated serum, as shown by Table II.

Several attempts were made to reactivate heated turtle serum with other sera, but these attempts all failed. Attempts to activate

human serum with small quantities of turtle serum gave no better results than when bouillon was used instead of human serum. In summing up, we can say that streptococci multiply rapidly in the blood sera of most cold-blooded animals. Turtle serum has considerable bacteriolytic power over these organisms, and it would seem that this property is connected with the presence of a special complement.

THE ACTION OF CERTAIN SALTS ON THE COMPLEMENT IN IMMUNE SERUM.*

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IN a recent article¹ Dr. Hektoen has pointed out that certain salts possess the property of suspending the hemolytic action of normal human serum on rabbit's corpuscles and its bacteriolytic action on typhoid bacilli. He has further shown that this suspension is probably due to the action of certain products of the ionization of these salts on the complement in the serum. At his suggestion, I have undertaken to determine whether or not a similar suspension takes place in an artificially immunized serum, and, if so, to inquire somewhat further into the chemistry of the process.

The work forming the basis for this report was done at the University of Chicago during the summer of 1903. Limitations in time have necessarily left it incomplete in many of its phases, but enough has been accomplished to show that there is the possibility of much valuable—and perhaps practical—information coming from a further pursuance of this line of investigation.

Material and apparatus.—The serum used for the study was obtained by immunizing a goat against dog's corpuscles, by weekly intraperitoneal injections of defibrinated dog's blood. One week after the third injection the serum was strongly hemolytic to dog's corpuscles. A record of the injections and hemolytic powers is shown in Table I.

To obtain the serum, blood was drawn from the jugular vein of the goat by means of the apparatus described by Portis on p. 129 of this issue. The blood was allowed to coagulate, and was then put in a refrigerator till the serum separated. This usually took place in about two hours. The serum thus obtained was generally perfectly clear. The few corpuscles that sometimes

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¹ *Trans. of the Chi. Path. Soc.*, 1903, 5, 303-309.

remained in it were removed by centrifugation. The dog's blood was drawn in a somewhat similar apparatus, was defibrinated by beating it with a sterile glass rod, and placed in a refrigerator till used. In drawing blood, of course, every aseptic precaution was taken.

TABLE I.
Record of Injections and Hemolytic Power of Serum.

DATE	INJECTIONS	HEMOLYTIC POWER ²		REMARKS
		Unwashed Corpuscles	Washed Corpuscles	
1903				
June 30.....	20 c.c. dog's blood	0+%	..	
July 6.....	25 c.c. dog's blood	6	..	
13.....	30 c.c. dog's blood	25	..	
20.....	35 c.c. dog's blood	95	..	Marked agglutination
August 3....	20 c.c. dog's blood	...	55%	
17....	20 c.c. dog's blood	...	55	
24	Unstable c'mplem'nt ³
29....	30 c.c. dog's blood	100	60	
September 7.	57	Compl't again stable

During the earlier experiments considerable difficulty was experienced in handling the dog's corpuscles. These are very delicate, and often broke up spontaneously in physiological saline. To avoid this the following precautions were afterward taken:

1. Blood was taken when possible only from strong, robust dogs, apparently in good health and spirits.

2. Blood was drawn in much larger quantities than needed for the experiments. It was found that larger quantities could be defibrinated with greater ease and apparently with less harm to the corpuscles.

3. The corpuscles were allowed to stand for an hour before being separated from the serum. This was for the purpose of

² Measured according to the scale proposed by WENSTRAND and described by him in the *Trans. of the Chi. Path. Soc.*, 1903, 5, 288-298.

³ Serum drawn on the afternoon of August 17 gave, the next morning, 55 per cent. hemolysis with washed corpuscles and 20 per cent. hemolysis after it had been standing in the refrigerator for four days. Serum drawn August 24, August 26, and August 29 almost completely lost its hemolytic power for washed corpuscles in twenty-four hours, and completely lost it in two days. The hemolytic power for unwashed corpuscles, however, remained intact, showing that the loss was probably due to a rapid degeneration of the complement, the dog's complement being capable of reactivating the serum. A week later the normal keeping quality of the serum had returned. No reason could be found, either in technic or care of animal, to account for this temporary instability of the complement.

allowing the bactericidal substances in the serum time to act on any possible contaminating organisms.

4. In handling the corpuscles, all unnecessary shaking, stirring, etc., was avoided. It was found that rough treatment readily broke up the corpuscles.

5. The blood was generally used within two hours of its time of withdrawal, never later than three hours.

With these precautions it was found possible to keep dog's corpuscles in physiological saline for several days without more than a slight yellow tint appearing in the supernatant liquid, a tint that is recorded in subsequent experiments as 0+ per cent. hemolysis.

The salt solutions used in the investigation were all of the same concentration, one-eighth gram molecular ($\frac{m}{8}$). They were made from chemically pure salts and water redistilled from glass. Before being used the solutions were sterilized in an autoclave (115°C. for seven minutes), care being taken to make the necessary evaporation as small as possible and uniform for the various solutions. All glassware used in the work was rendered chemically clean by a long treatment with alkali, acid, and distilled water, was thoroughly dried, plugged with absorbent cotton, and sterilized by dry heat (140°C. for one hour).

Method.—To test the effect of a salt solution on the hemolytic power of the serum, varying amounts of the solution were measured out into small test-tubes (3 c.c.), enough physiological saline added to make the volumes up to a uniform amount (1.0 c.c.), and a given quantity of the serum (0.2 c.c.) added to each. The strictest aseptic technic was, of course, employed. The resulting mixtures were thoroughly shaken and allowed to stand for an hour to permit a thorough interaction of the salt and serum. A uniform amount (0.5 c.c.) of a 20 per cent.⁴ suspension of washed dog's corpuscles was then added to each mixture and the tubes incubated at 37.5°C. for two hours. They were then placed in a refrigerator, and about fifteen hours later the amount of hemolysis estimated from the depth of color in the clear liquid above the settled corpuscles.

⁴ Five times the original volume of blood.

With the serum used agglutination was so prompt that, if measures had not been taken to avoid it, the corpuscles would have been clumped in a few minutes at the bottom of the tubes, and thus, to a certain extent, removed from the action of the serum. To prevent this, the test-tubes in the earlier experiments were sealed with sterile paraffin and placed on their sides, so that the corpuscles might agglutinate along the length of the tubes, in a form more accessible to chemical action. In the later experiments a rack was used in which the tubes could be placed in a nearly horizontal position, without sealing. After incubation the tubes were placed in an upright position.

In order to estimate the amount of hemolysis, a color scale was prepared in each experiment by laking the corpuscles used in that experiment in distilled water, in such proportions that 100 per cent. on the scale meant a complete solution of all the corpuscles in each test-tube.

Effects of salts on corpuscles.—According to the above method, the corpuscles were submitted, not only to the action of the modified serum, but to the action of the salt solutions as well. It was necessary, therefore, to know the hemolytic powers of the salt

TABLE II.

Effect of Salt Solutions on Dog's Corpuscles.

(Recorded amounts of salt solution made up to 1.2 c.c. with physiological saline; 0.5 c.c. of a 20 per cent. suspension of washed dog's corpuscles added; incubated for two hours, refrigerator fifteen hours. Record shows percentage of hemolysis.)

SOLUTION	CaCl ₂	BaCl ₂	Na ₂ C ₂ O ₄	Na ₂ SO ₄	MgCl ₂	Na ₂ C ₂ H ₃ O ₆	NaI	NaNO ₃	KCl	NH ₄ Cl	Na ₂ CO ₃	Na ₃ PO ₄
	%	%	%	%	%	%	%	%	%	%	%	%
0.00 c.c. $\frac{m}{s}$ solution.	0+	0+	0+	0+	0+	0+	0+	0+	0+	0+	0+	0+
0.05 c.c. "	7-	0+	0+	5-	0+	0+	0+	0+	5-	0+	100	100
0.1 c.c. "	15	0+	0+	0+	5-	0+	0+	0+	5	5-	100	100
0.15 c.c. "	20	0+	0+	0+	5-	0+	0+	0+	5	5-	100	100
0.2 c.c. "	25	0+	0+	0+	5	0+	0+	0+	5+	5-	100	100
0.3 c.c. "	30	0+	0+	5-	7-	0+	0+	0+	5+	5-	100	100
0.4 c.c. "	30+	0+	0+	0+	7-	0+	0+	0+	7-	5	100	100
0.5 c.c. "	35	0+	0+	0+	7-	0+	0+	0+	7-	5	100	100
0.6 c.c. "	35	0+	0+	0+	7-	0+	0+	0+	10-	5+	100	100
0.7 c.c. "	40-	0+	0+	0+	7-	5-	0+	0+	10	20	100	100
0.8 c.c. "	40	0+	0+	5-	7-	0+	0+	0+	10	20	100	100
0.9 c.c. "	40+	0+	5-	0+	7+	5-	0+	5-	10	80	100	100
1.0 c.c. "	45	0+	5-	0+	10-	5-	5-	5-	10+	100	100	100

solutions alone, before attempting to judge their effects on the hemolytic powers of the serum. To determine this, corpuscles were exposed to the action of the salt solutions, the tubes containing them incubated for two hours, placed in a refrigerator, and the percentage of hemolysis estimated colorometrically fifteen hours later, exactly as in the subsequent serum experiments. The results of a number of such experiments are shown in Table II. From this it is seen that, under the conditions of the experiment, BaCl_2 , $\text{Na}_2\text{C}_2\text{O}_4$, Na_2SO_4 , $\text{Na}_2\text{C}_2\text{H}_4\text{O}_6$, NaI , and NaNO_3 are practically without hemolytic effect on dog's corpuscles; that KCl and MgCl_2 produce slight hemolysis; and that CaCl_2 , NH_4Cl , Na_2CO_3 , and Na_3PO_4 , have strong hemolytic powers.

Effects of salts on serum.—Experiments were now undertaken to determine the effects of these solutions on the hemolytic powers

TABLE III.

Effect of Salt Solutions on the Hemolytic Action of Immune Serum.

(Recorded amounts of salt solution made up to 1.0 c.c. with physiological saline, 0.2 c.c. of hemolytic serum added, and the mixture allowed to act on 0.5 c.c. of a 20 per cent. suspension of washed dog's corpuscles; incubated for two hours, refrigerator fifteen hours. Record shows percentage of hemolysis.)

SOLUTION	CaCl_2	BaCl_2	$\text{Na}_2\text{C}_2\text{O}_4$	Na_2SO_4	MgCl_2	$\text{Na}_2\text{C}_2\text{H}_4\text{O}_6$	NaI	NaNO_3	KCl	NH_4Cl	Na_2CO_3	Na_3PO_4
	%	%	%	%	%	%	%	%	%	%	%	%
0.00 c.c. solution..	45	50	55	50	50	45	50	50—	45	45	45	50
0.05 c.c. " . . .	40+	40	50	50	45	30+	50	50	50—	45+	100	100
0.1 c.c. " . . .	25	30+	45	50+	40	35	45	50	45	50	100	100
0.15 c.c. " . . .	20+	25	35	40	35	30	45	50+	45+	50	100	100
0.2 c.c. " . . .	20	20	20	25	30	20+	55—	50	50	50	100	109
0.3 c.c. " . . .	7	5	5	20	25	25	50+	50	50	50+	100	100
0.4 c.c. " . . .	0+	0+	0+	15	20+	20—	45+	50+	55	60	100	100
0.5 c.c. " . . .	0+	0+	0+	10—	15	20	45	50	60—	60+	100	100
0.6 c.c. " . . .	0+	0+	0+	5+	10+	10+	45	45	60—	80	100	100
0.7 c.c. " . . .	0	0	0+	5	10	10+	45	45	60	100—	100	100
0.8 c.c. " . . .	0	0	0+	5	10	10+	45	45	60	100	100	100
0.9 c.c. " . . .	0	0+	0+	5	10—	10—	47	45	65	100	100	100
1.0 c.c. " . . .	0	0+	0+	0+	5+	7	50	45	65+	100	100	100

of the serum. The salt solutions and serum were mixed in varying proportions and the mixture allowed to act on corpuscles in the manner described above. The results are recorded in Table III. From this table it is seen that CaCl_2 , BaCl_2 , and $\text{Na}_2\text{C}_2\text{O}_4$ possess the property of rapidly destroying or inhibiting the

hemolytic powers of the serum—a property that is possessed also by Na_2SO_4 , MgCl_2 , and $\text{Na}_2\text{C}_2\text{H}_4\text{O}_6$, though to a less degree. As to the action of Na_2CO_3 and Na_3PO_4 no conclusion can be drawn, as these salts in themselves are such powerful hemolytic agents. KCl and NH_4Cl are probably without effect on the hemolytic power, as the percentage of hemolysis obtained with each mixture is, within the limits of error in such experiments, approximately equal to that of the serum itself plus that of the salt solution. Experiments with NaI and NaNO_3 were contradictory, one series showing a slight suspension in hemolytic power, another a slight increase. The recorded results are the averages of two such contradictory series.

Having thus determined that certain salts have the power of suspending the hemolytic action of the serum, the inquiry was next directed to the manner in which they bring about this result. It is evident that there are four ways in which they might do this: (*a*) they might alter the blood corpuscle, physically or chemically, in such a way as to render it insusceptible to the hemolytic action of the serum; (*b*) they might destroy the hemolytic amboceptor, or alter it chemically in such a way as to prevent its union with the corpuscle or with the complement, or otherwise destroy its efficiency in hemolytic action; (*c*) they might destroy the hemolytic complement, or alter it chemically in such a way as to prevent its union with the amboceptor or otherwise destroy its efficiency; and (*d*) it is even conceivable that they might prevent hemolysis by some purely physical action, unassociated with chemical changes in either complement or amboceptor. Experiments were undertaken to test each of these possibilities.

a) Do the salts render the blood corpuscles insusceptible to the hemolytic action of the serum? To test this, washed corpuscles were exposed to the action of the various salt solutions for a couple of hours, were then separated from the solutions by centrifugation, suspended in physiological saline, and submitted to the action of the serum. The results are shown in Table IV. From this it is seen that corpuscles treated with the various salts gave practically the same amount of hemolysis as untreated corpuscles. Unless it is conceivable that the process of centrifuga-

tion and subsequent suspension in physiological saline might undo the action of these salts—a conception that further experiments proves to be without foundation—it follows from this that the prevention of hemolysis by these solutions is not due to alterations produced by them in the blood corpuscles.

TABLE IV.

The Effect of Salt Solutions on the Hemolytic Susceptibility of Dog's Corpuscles.

(0.5 c.c. of a 20 per cent. suspension of dog's corpuscles⁵ exposed to the action of recorded amounts of salt solution for two hours; corpuscles then separated by centrifugation and submitted to the action of 0.2 c.c. of hemolytic serum. Record shows percentage of hemolysis.)

SALT SOLUTION	SUBSEQUENT HEMOLYSIS		
	Reading 1	Reading 2	Average
0.5 c.c. $\frac{m}{8}$ CaCl_2 + 0.5 c.c. physiol. saline.	55%	50%	52.5%
0.5 c.c. $\frac{m}{8}$ BaCl_2 + 0.5 c.c. physiol. saline.	55—	55—	55—
0.5 c.c. $\frac{m}{8}$ $\text{Na}_2\text{C}_2\text{O}_4$ + 0.5 c.c. phy. saline.	50	55	52.5
1.0 c.c. $\frac{m}{8}$ Na_2SO_4	55	50	52.5
1.0 c.c. $\frac{m}{8}$ MgCl_2	55	60+	58
1.0 c.c. $\frac{m}{8}$ $\text{Na}_2\text{C}_2\text{H}_4\text{O}_6$	50	50	50
Untreated corpuscles.....	50—	55	52

b) Do the solutions destroy the hemolytic amboceptor, or alter it chemically in such a way as to prevent its union with the corpuscle or with the complement, or otherwise destroy its efficiency in hemolytic action? To answer this, hemolytic serum was mixed with the salt solutions in proportions that had been found to suspend hemolysis, and the mixtures allowed to stand, at room temperature, for one and one-half hours. These mixtures were then cooled to 0° C., and at this temperature allowed to act on washed corpuscles.

Normally, at this temperature, the amboceptor unites with the corpuscles, but the complement, possessing a less affinity, does not unite with the amboceptor. As a result no hemolysis takes

⁵ The experiments, of course, were done on a much larger scale than this, the table recording simply the amounts that entered into each test-tube.

place, but the corpuscles become sensitized (corpuscle + amboceptor), so that a subsequent exposure of them to pure complement, which in itself possesses no hemolytic power, leads to their solution.

At the end of forty minutes, the corpuscles so exposed were separated from the serum-salt mixtures by centrifugation and washed repeatedly in physiological saline, care being taken to

TABLE V.

The Effect of Salt Solutions on the Hemolytic Amboceptor.

(0.2 c.c. hemolytic serum added to recorded amounts of salt solution, mixtures allowed to stand one and one-half hours at room temperature, then used to sensitize 0.5 c.c. of a 20 per cent. corpuscle suspension. Corpuscles separated by centrifugation and submitted to the action of pure complement [non-immune goat's serum]. Record shows percentage of hemolysis.)

SALT SOLUTION	SUBSEQUENT HEMOLYSIS BY PURE COMPLEMENT		
	Reading 1	Reading 2	Average
0.5 c.c. $\frac{m}{s}$ CaCl_2 + 0.5 c.c. physiol. saline.	45%	50%	47.5%
0.5 c.c. $\frac{m}{s}$ BaCl_2 + 0.5 c.c. physiol. saline.	40	55	47.5
0.5 c.c. $\frac{m}{s}$ $\text{Na}_2\text{C}_2\text{O}_4$ + 0.5 c.c. phy. saline.	50	50	50
1.0 c.c. $\frac{m}{s}$ Na_2SO_4	50	45	47.5
1.0 c.c. $\frac{m}{s}$ MgCl_2	50	55	52.5
1.0 c.c. $\frac{m}{s}$ $\text{Na}_2\text{C}_2\text{H}_4\text{O}_6$	55	50	52.5
Untreated serum	50	50	50

(Pure complement gave with untreated corpuscles 5 + per cent. hemolysis. Sensitized corpuscles not treated with complement gave in each case approximately 5 per cent. spontaneous laking.)

keep the temperature as near 0° C. as possible during the process. The treated corpuscles were then submitted to the action of pure complement (normal goat's serum). The results are recorded in Table V. In all cases practically the same amount of hemolysis took place as in the control experiment with serum not treated with a salt solution. The various salt-serum mixtures are, therefore, capable of sensitizing corpuscles to the same degree as untreated serum. This not only confirms our conclusion of the last experiment that the solutions produce no changes in the corpuscle

preventing the union of the amboceptor, but also proves that they do not alter the amboceptor in such a way as to prevent its union with the corpuscle. Unless the subsequent suspension of the sensitized corpuscle in physiological saline undoes the action of these salts on the amboceptor attached to the corpuscle, it also follows that the solutions do not alter the amboceptor in such a way as to prevent its union with the complement, or its fulfilling its other functions in hemolytic action.

TABLE VI.

Effect of Salt Solutions on the Hemolytic Complement.

(0.2 c.c. pure complement [normal goat's serum] added to recorded amounts of salt solution; mixtures allowed to stand one and one-half hours at room temperature, and then allowed to act on 0.5 c.c. of a 20 per cent. suspension of sensitized corpuscles. Record shows percentage of hemolysis.)

SALT SOLUTION	SUBSEQUENT ACTION ON SENSITIZED CORPUSCLES		
	Reading 1	Reading 2	Average
0.5 c.c. $\frac{m}{s}$ CaCl_2 + 0.5 c.c. physiol. saline.	5+	10%	8%
0.5 c.c. $\frac{m}{s}$ BaCl_2 + 0.5 c.c. physiol. saline.	10—	5+	7.5
0.5 c.c. $\frac{m}{s}$ $\text{Na}_2\text{C}_2\text{O}_4$ + 0.5 c.c. phy. saline.	5+	10—	7.5
1.0 c.c. $\frac{m}{s}$ Na_2SO_4	15—	10	12
1.0 c.c. $\frac{m}{s}$ MgCl_2	10—	10+	10
1.0 c.c. $\frac{m}{s}$ $\text{Na}_2\text{C}_2\text{H}_4\text{O}_6$	10+	10—	10
Untreated complement.....	85+	90	88

(Sensitized corpuscles not treated with complement gave 7 per cent. spontaneous laking.)

c) The evidence, therefore, indirectly points either to the complement as being the seat of chemical action, or to the action being a purely physical one. To obtain direct evidence on this point, pure complement was mixed with the salt solutions, and this treated complement afterwards allowed to act on sensitized corpuscles, a control being made with untreated complement. The results are recorded in Table VI. From this it is seen that the sensitized corpuscles gave with the untreated complement 88 per cent. hemolysis, but that with the treated complement there was in each case but a trifle more hemolysis than the spontaneous

laking of the sensitized corpuscles (7 per cent.). The experiment furnishes direct evidence that the action of the salts is on the complement, though here again the possibilities of the action being a physical one, or of its being on the amboceptor attached to the sensitized corpuscle, are not ruled out.

In order to rule out definitely the amboceptor as the point of attack, it was attempted to vary the amount of complement in a serum without changing the amount of amboceptor, and to deter-

TABLE VII.

Relation between Amount of Complement and Amount of CaCl_2 Necessary to Suspend Hemolysis.

(0.2 c.c. of heated hemolytic serum [pure amboceptor] + recorded amounts of non-immune serum [pure complement] added to recorded amounts of CaCl_2 and the volumes made up to 1.5 c.c. by the addition of physiological saline. This mixture allowed to act on 0.5 c.c. of a 20 per cent. suspension of dog's corpuscles. Record shows percentage of hemolysis.)

AMOUNT OF COMPLEMENT	0.00 c.c. m 8 CaCl_2	0.05 c.c. m 8 CaCl_2	0.1 c.c. m 8 CaCl_2	0.15 c.c. m 8 CaCl_2	0.2 c.c. m 8 CaCl_2	0.3 c.c. m 8 CaCl_2	0.4 c.c. m 8 CaCl_2	0.5 c.c. m 8 CaCl_2	0.6 c.c. m 8 CaCl_2	0.7 c.c. m 8 CaCl_2	0.8 c.c. m 8 CaCl_2	0.9 c.c. m 8 CaCl_2	1.0 c.c. m 8 CaCl_2
0.1 c.c. non-immune serum (pure complement)	30	15	10	0+	0	0	0	0	0	0	0	0	0
0.2 c.c. "	50	40	35	20	15	0+	0	0	0	0	0	0	0
0.3 c.c. "	70	60-	50	40	30	10+	5	0+	0	0	0	0	0

mine for this variable serum the minimum amount of salt necessary to suspend hemolysis.⁶ If the amboceptor is the point of attack, the amount of salt should remain constant, no matter how the complement varies. If, on the other hand, the complement is the point of attack, the amount should vary with variations in the complement.

Sera with different complement content but constant amboceptor were made by adding to heated hemolytic serum (pure amboceptor) varying quantities of normal goat's serum (pure complement). The results are recorded in Table VII. From this it is seen that with one unit of complement 0.15 c.c. $\frac{m}{8}$ CaCl_2

⁶ A second series with constant complement but varying amboceptor was planned, but the time was too short to carry this out.

was required to suspend hemolysis, that with two units of complement 0.3 c.c. $\frac{m}{8}$ CaCl_2 was required, and that with three units 0.5 c.c. $\frac{m}{8}$ CaCl_2 . The ratio of the amounts of complement, 1 : 2 : 3, is approximately equal to that of the CaCl_2 , 0.15 : 0.3 : 0.5 ($=1 : 2 : 3\frac{1}{3}$), and differs from it not more than the errors of observation warrant. It is evident, therefore, that the amboceptor cannot be the point of attack, and that the salts either act chemi-

TABLE VIII.

Relation between Age of the Serum and Amount of BaCl_2 Necessary to Suspend Hemolysis.

(0.2 c.c. of serum was exposed on successive days to the action of recorded amounts of BaCl_2 and the mixtures allowed to act on 0.5 c.c. of a 20 per cent. corpuscle suspension. Record shows percentage of hemolysis.)

AGE OF SERUM	0.00 c.c. $m/8$ BaCl_2	0.05 c.c. $m/8$ BaCl_2	0.1 c.c. $m/8$ BaCl_2	0.15 c.c. $m/8$ BaCl_2	0.2 c.c. $m/8$ BaCl_2	0.3 c.c. $m/8$ BaCl_2	0.4 c.c. $m/8$ BaCl_2	0.5 c.c. $m/8$ BaCl_2	0.6 c.c. $m/8$ BaCl_2	0.7 c.c. $m/8$ BaCl_2	0.8 c.c. $m/8$ BaCl_2	0.9 c.c. $m/8$ BaCl_2	1.0 c.c. $m/8$ BaCl_2
Serum 1 hour old (Aug. 17).....	100+	100	90+	70	60	40	35	25	0+	0+	0	0	0
Serum 1 day old.....	60	60	50	40+	35	7	5-	0+	0	0	0	0	0
Serum 3 days old.....	40	25	15+	10	5-	0+	0	0	0	0	0	0	0
Serum 7 days old.....	20	10	5	0+	0+	0	0	0	0	0	0	0	0

cally on the complement or produce their results by purely physical changes.

It might be objected to the above experiment, however, that the complement was not the only variable substance in the serum, that, with the extra amounts of normal serum, extra amounts of serum-albumin, serum-globulin, and the like were added. To avoid this criticism, a second series was run in which the volume of the serum mixtures was made up to a uniform amount by the addition of heated, normal goat's serum (containing neither complement nor amboceptor, but containing serum-albumin, serum-globulin, etc.). The results of such an experiment correspond roughly with those recorded above, though here less difference was noted between the amounts of CaCl_2 needed with the different amounts of complement. A more convincing experiment, however,

was made by testing the same serum on successive days, as the complement degenerated, the amboceptor, presumably, remaining constant during the short time over which the experiments extended. The result of such an experiment is recorded in Table VIII. Here again the same general law holds good that, with variation in the amount of complement, there is a parallel variation in the amount of salt necessary to suspend hemolysis.

We have, therefore, definitely proved that the salt solutions suspend hemolysis either by chemical changes produced by them in the complement, toward which the above experiments strongly point, or by possible physical changes in the liquid. Experiments were next undertaken to determine the fate of the complement in this suspension. Is the complement broken up by the salts, or is the suspension accomplished without its destruction? To determine which of these is true, the inhibiting salt was precipitated from non-hemolytic, serum-salt mixtures, and the hemolytic powers of the remaining liquids tested. The results are recorded in Table IX. In all cases the hemolytic power was restored by such precipitation, and, with two exceptions, practically to the original amount possessed by the serum.

To test whether this return of hemolytic power was due to a liberation of the complement or to possible products of the interaction of the salts used, the liquids were now heated to 56° C. for forty minutes and the hemolytic power again tested. Except in the experiments in which MgCl_2 entered, the hemolytic power was destroyed by such heating, showing that, with these two exceptions,⁷ the restored power was due to the presence of a thermo-labile substance, undoubtedly the original complement. The experiments, therefore, prove that the suspension of hemolysis is accomplished without destruction of the complement.

d) Evidence ruling out the possibility of the action being a physical one is obtained by comparing the results recorded in

⁷ An examination of the MgCl_2 solution showed that, on precipitating the Mg ion with $\text{Na}_2\text{C}_2\text{H}_3\text{O}_6$, there was left behind in the liquid a strong thermo-stable, hemolytic agent, the exact nature of which was not determined, but which probably came from a contamination in the MgCl_2 solution. It has been suggested that in the process of autoclaving small quantities of HCl might have been driven off from this solution, leaving behind $\text{Mg}(\text{OH})_2$, minute traces of which are soluble. On precipitating this with a Na salt, NaOH, a powerful hemolytic agent, would be formed. Time was too limited to test the truth of this suggestion.

TABLE IX.
Effect of Precipitating the Inhibiting Salt.

SÉRUM	PERCENTAGE OF HEMOLYSIS				HEMOLYSIS AFTER ADDING SALT SOLUTION				PRECIPITANT ^s	HEMOLYSIS AFTER PRECIPITATION				SAME AFTER HEATING		
	Reading 1	Reading 2	Reading 3	Average	Reading 1	Reading 2	Reading 3	Average		Reading 1	Reading 2	Reading 3	Average	Reading 1	Reading 2	Average
0.2 c.c. serum	70	60	65	65%	0.3 c.c. $\frac{m}{8}$ CaCl ₂	7	7	5+	6.3%	0.3 c.c. $\frac{m}{8}$ Na ₂ C ₂ O ₄	60	70	60	63.3%	0	0
										0.3 c.c. $\frac{m}{8}$ Na ₂ C ₂ H ₄ O ₆	65	65	65	0	0	0
					0.3 c.c. $\frac{m}{8}$ BaCl ₂	7	7	7	7	0.3 c.c. $\frac{m}{8}$ Na ₂ C ₂ O ₄	65	70	60	65	0	0
										0.3 c.c. $\frac{m}{8}$ Na ₂ C ₂ H ₄ O ₆	65	70	65	66.7	0	0
					0.3 c.c. $\frac{m}{8}$ Na ₂ C ₂ O ₄	10	15	10	11.7	0.3 c.c. $\frac{m}{8}$ Na ₂ SO ₄	60	55+	65	60	0	0
0.1 c.c. serum	30+	30	40	33.3	0.45 c.c. $\frac{m}{8}$ Na ₂ C ₂ H ₄ O ₆	5+	5	5+	5+	0.45 c.c. $\frac{m}{8}$ CaCl ₂	25+	30	30	28.3	0	0
										0.45 c.c. $\frac{m}{8}$ BaCl ₂	30	35	30	31.7	0	0
					0.45 c.c. $\frac{m}{8}$ Na ₂ SO ₄	7	7	7	7	0.45 c.c. $\frac{m}{8}$ BaCl ₂	30	35	30	31.7	0	0
					0.45 c.c. $\frac{m}{8}$ MgCl ₂	20	20—	20+	20	0.45 c.c. $\frac{m}{8}$ Na ₂ C ₂ O ₄	70	80	70	73.3	40	45
																42.5

^sWith several of the solutions it is necessary to start the process of precipitation by scratching the inside of the test tube with a sterile glass rod.

Tables II and III. From these tables it is seen that CaCl_2 possesses strong hemolytic powers, but that, mixed with serum, it not only prevents the hemolytic action of the serum, but loses its own power of hemolysis. It is inconceivable that this could be brought about as long as the CaCl_2 remained free in the solution. It is probable, therefore, that the CaCl_2 , or its active ion, is in some way removed by the serum from an active rôle in the liquid. This would be accomplished if it were bound chemically to the complement.

Further evidence that such a chemical union takes place is furnished by the fact that on heating a non-hemolytic CaCl_2 serum mixture, the complement is not only destroyed, as can be proved by the precipitation method described above, but that at the same time, the hemolytic power of the CaCl_2 is, in large part, restored. The evidence, therefore, is very strong that the suspending action of the salts is not due to physical changes, but to chemical alterations in the complement.

Since the complement is not broken up by the salts, this action is probably the formation of simple salt or ion compounds with the complement, which compounds are either incapable of union with the amboceptor or hemolytically inactive after such a union.

The salts investigated are all electrolytes, and in the dilutions used are largely ionized. There is reason to believe, therefore, that their action is due to the ions Ca^{++} , Ba^{++} , Mg^{++} , SO_4^{--} , $\text{C}_2\text{O}_4^{--}$, $\text{C}_2\text{H}_4\text{O}_6^{--}$, their solutions contain, rather than to the salt molecules as a whole. It would be very difficult, however, to prove beyond a question of doubt that such is the case.

CONCLUSIONS.

1. Certain salts, among which are CaCl_2 , BaCl_2 , MgCl_2 , Na_2SO_4 , $\text{Na}_2\text{C}_2\text{O}_4$, and $\text{Na}_2\text{C}_2\text{H}_4\text{O}_6$, possess the property of inhibiting the hemolytic action of an artificially immunized goat's serum on dog's corpuscles.

2. These salts produce no changes in the corpuscles or in the amboceptor that interfere with hemolytic action.

3. The amount of salt necessary to bring about the inhibition varies with the amount of hemolytic complement in the serum.

4. Precipitation of the inhibiting salts from non-hemolytic, serum-salt mixtures causes a complete return of hemolytic power, which power is destroyed by heating the resulting liquid to 56° C. for forty minutes.

5. There is reason, therefore, to believe that the inhibition is due to the formation of simple salt- or ion-complement compounds, which are either incapable of uniting with the amboceptor or hemolytically inactive after such union.

EXPERIMENTAL STUDY OF THYROTOXIC SERUM.*

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INTRODUCTION.

It is well known that complete removal of the thyroid gland is followed by a typical group of symptoms, terminating in death. More recently it has been shown by a number of observers that the acute symptoms, simulating tetany, and the rapid fatal termination follow the removal of the parathyroid bodies, even though the entire thyroid is left *in situ*. On the other hand, the removal of the thyroid, leaving the parathyroids untouched, is followed by chronic symptoms, such as myxedema, cretinism, and cachexia.

The present work was begun in the hope that it might be possible to produce similar clinical pictures by means of specific cytotoxins, selective in their action on the thyroid and parathyroid cells.

Preliminary reports of Mankovsky¹ and Gontscharukov² lent encouragement to this view. Both stated that they had produced thyrotoxic sera, injections of which produced symptoms simulating tetany, the thyroid cells showing microscopic evidences of degeneration. Mankovsky introduced the thyroids of dogs into the peritoneal cavity of cats, at intervals of two weeks, and after the third injection bled the cats and separated their serum, which proved toxic to dogs.

* Received for publication October 15, 1903.

¹ *Russ. Arch. f. Path.*, 1902, 14, 571-591.

² *Centralbl. f. allg. Path. u. path. Anat.*, 1902, 13, 121-124.

Gontscharukov also used the thyroids of dogs, injecting them into rams. He, too, produced a serum which he concluded was thyrotoxic. Neither of these workers, so far as I can learn, has published a subsequent full report, and as their experiments were conducted on a few dogs only, their work cannot be regarded as conclusive.³

While many problems have arisen during the progress of my experiments that demand much more study than I have been able to give them, the results so far obtained appear of enough importance to merit publication at this time.

METHODS OF IMMUNIZATION.

Goats and dogs were used for my experiments. At first an emulsion of dogs' thyroids was prepared and injected into the peritoneal cavity of goats. The thyroids were removed, with all aseptic precautions, cut up into small pieces, and placed in a mortar containing some purified sand. The sand was prepared by digesting with weak hydrochloric acid for twenty-four hours, to remove carbonates and render other salts soluble, then washed repeatedly with distilled water until all traces of the acid were gone, and finally incinerated to destroy all organic matter.

On account of the tough, fibrous nature of the thyroid, it was found necessary to continue the grinding for several hours; to prevent contamination, the mortar was covered with a sheet of asbestos, perforated to allow the passage of the pestle. When the mass was completely pulpified, a small amount of sterile salt solution (0.85 per cent.) was added and the mixture stirred. The supernatant portion was pipetted off to get rid of the sand, and then strained through a fine wire gauze to remove the larger pieces of tissue. One obtains in this manner an emulsion containing not only a preponderance of thyroid cells, but also a small amount of various cells, common to all tissues.

Increasing doses of emulsion were injected into the peritoneal cavity of goats, at intervals of ten to fourteen days; at first one dog's thyroid was used, then two, and so on, until finally ten thyroids were used for a single injection. After the third injection, the study of the sera of the immunized animals was begun. The immunization was kept up, never allowing an interval of more than one month to elapse between injections. Aside from a slow progressive loss in weight, no effect was noticed on the goats.

Later in the work it was thought best to remove all of the blood from the thyroids before making an emulsion, and this was readily accomplished by washing out the gland *in situ*, through the thyroid artery, using a sterile

³The report by J. L. YATES (*Univ. of Penna. Med. Bull.*, 1903, 16, 195-200) on the experimental production of cytolytins for the adrenal, thyroid, and parathyroid glands of dogs appeared after the present report had been placed in the hands of the editors. Also the report of W. G. MACCALLEM (*Med. News.*, Oct. 31, 1903), "On the Production of Specific Cytolytic Sera for Thyroid and Parathyroid."

normal salt solution. The washing was continued until the gland became completely bloodless. That this was accomplished was apparent also in microscopic sections of washed thyroids. One of the goats was immunized with injections of washed thyroids.

For purposes of comparison, one goat was immunized with an extract of dog's thyroid, known as "colloid matter." This was prepared according to Hutchinson's method, by digesting sterile crushed thyroids with sterile magnesium sulphate solution (5 per cent.) for six hours. Then the mixture was thoroughly centrifugalized to throw down all undissolved matter, and the clear supernatant liquid pipetted off. To this fluid a few drops of acetic acid were added, and a heavy whitish flocculent precipitate was thrown down, which was separated and washed to remove all of the acetic acid. A suspension of this precipitate—the colloid matter—was made in normal salt solution, and injected into the peritoneal cavity of a goat at intervals of ten days, using an increasing number of thyroids, as in the previous experiments.

Method of bleeding.—As considerable difficulty was met with in bleeding the goats, a special Erlenmeyer flask, with a small glass tube blown into its side near the neck, and another at the bottom, was used. To the arm at the bottom an inspirating needle is fastened, and capped with a test-tube. The upper arm is plugged with cotton, and serves as the place of attachment for the aspirating syringe. The flask is fitted with a rubber cork, and to keep the clot from sliding about two glass rods are inserted. The blood is easily withdrawn from the superficial jugular vein, which stands out prominently, with a little pressure at the side of the neck, near the anterior border of the sterno-cleido-mastoid muscle. The goats stand the bleeding very well, and as much as 400 c.c. were withdrawn at one time. The blood clots very quickly, and the serum is easily removed by slipping the needle off and pouring the serum into sterile flasks.

EXPERIMENTS WITH THE SERUM OF GOATS IMMUNIZED WITH DOGS' THYROIDS.

For control purposes, several dogs were given intravenous injections of normal goat serum, and as much as 7 c.c. per kilogram weight was found without any effect. The animals were not at all depressed, showed no abnormal symptoms, and when killed one or two weeks later there were no microscopic changes anywhere.

The dogs injected with the immune sera may be divided into three groups. Group I (see Table I) received injections of serum from goats immunized with thyroid tissue; Group II (see Table II) received injections of the same serum as the former, but it was first treated with dogs' red blood corpuscles to remove the hemolytic amboceptors; and finally Group III (see Table III) consisting of dogs injected with serum from the goat immunized with "colloid matter."

TABLE I.

Dogs Injected with Serum of Goats Treated with Dog's Thyroid.

Dog No.	Weight	Dose of Toxic Serum per Kilogram	Reinjected	Results
1	9 kg.	Jan. 22, 3.5 c.c. per kg., intravenous.		Nausea, vomiting, defecation, urination, and marked depression; <i>hemoglobinuria</i> . Lived ten hours.
3	5 kg.	Feb. 3, 2 c.c. per kg., intravenous.	Feb. 10, 3 c.c. per kg., intravenous.	After first injection, depression for several hours, but rallied. The next day very stupid, watering of eyes; <i>hemoglobinuria</i> ; slowly improved. After second injection, dog went into convulsions and died within one hour. Lived seven days after first injection.
4	7.5 kg.	Feb. 3, 2 c.c. per kg., intravenous.		Vomiting, frequent defecation, depression, watering of eyes, and stupid for several days; <i>hemoglobinuria</i> . Killed on sixth day.
5	3.5 kg.	Feb. 10, 3 c.c. per kg., intraperitoneal.		Shortly after injection, clonic convulsions which lasted several minutes; then animal walked about with marked spastic gait in hind legs. Next day, watering of eyes, frequent general tremors; quite sick; <i>hemoglobinuria</i> . Lived two days.
7	8 kg.	March 11, 3 c.c. per kg., intravenous.	March 17, 6 c.c. per kg., intravenous.	Depressed for a few days after first injection; watering of eyes; <i>hemoglobinuria</i> . After second injection, convulsions and death in two hours. Lived six days after first injection.
8	5.5 kg.	April 4, 4 c.c. per kg., intravenous.	April 5, 4 c.c. per kg.; April 11, 5 c.c. per kg.	Not very sick after first injection, but stupid and depressed for several days after second injection. After third, convulsions and death in one hour; <i>hemoglobinuria</i> . Lived seven days after first injection.
9	8 kg.	April 24, 4 c.c. per kg., intravenous.		Vomiting, frequent defecation; depressed and stupid; <i>hemoglobinuria</i> . Lived six hours.
10	7.5 kg.	April 29, 2 c.c. per kg., intravenous.		Not very sick; watering of eyes; slight <i>hemoglobinuria</i> . Killed on fifth day.
11	8 kg.	May 13, 1 c.c. per kg., intravenous.		Vomiting, and slight convulsions; then marked depression and death in eight hours. Lived eight hours.
13	6 kg.	May 20, 3 c.c. per kg., intravenous.	May 21, 5 c.c. per kg., intravenous.	Vomiting, frequent defecation, marked spastic gait in hind legs; watering of eyes; <i>hemoglobinuria</i> . After second injection, convulsions and death in one hour. Lived twenty-four hours after first injection.
15	9 kg.	May 25, 4 c.c. per kg., intravenous.	June 2, 4 c.c. per kg., intravenous.	Depressed and stupid for several days; watering of eyes; <i>hemoglobinuria</i> ; feces dark color. After second injection, convulsions and death in two hours. Marked loss in weight. Lived eight days after first injection.
17	9 kg.	June 3, 4 c.c. per kg., intravenous.	June 10, 5 c.c. per kg., intravenous.	Stupid and sick for several days; watering of eyes; <i>hemoglobinuria</i> ; feces dark red; then improved; but after second injection grew rapidly weak and cachectic, and died June 23. Lived twenty days after first injection.

TABLE I—*Continued.*

Dog No.	Weight	Dose of Toxic Serum per Kilogram	Reinjected	Results
19	5 kg.	June 24, 0.5 c.c. per kg., intravenous.	June 30, 1 c.c. per kg., intravenous.	Not much affected by first injection. After second, convulsions and death in thirty minutes; <i>hemoglobinuria</i> . Lived six days after first injection.
20	5.5 kg.	June 24, 4 c.c. per kg., intravenous.		Vomiting, frequent defecation, convulsions, and death in one hour. Lived one hour.
21	8 kg.	June 24, 2 c.c. per kg., intravenous.	June 30, 1 c.c. per kg., intravenous.	Depressed and stupid, and remained so; lost weight constantly, and became quite weak; <i>hemoglobinuria</i> ; watering of eyes. Died July 14. Lived twenty days after first injection.
23	7.5 kg.	June 30, 1.5 c.c. per kg., intravenous.		Vomiting, convulsions; death in thirty minutes.
24	8 kg.	June 30, 1 c.c. per kg., intravenous.	July 11, 1 c.c. per kg., intravenous.	Vomiting, diarrhea, depression; remained stupid for several days; watering of eyes; <i>hemoglobinuria</i> . After second injection, convulsions and death in one hour. Lived eleven days after first injection.
29	5 kg.	July 13, 7 c.c. per kg., intraperitoneal.		Vomiting, frequent defecation, depression. Found dead next morning—numerous subperitoneal ecchymoses, and some blood in peritoneal cavity; <i>hemoglobinuria</i> .
31	11 kg.	July 27, 7 c.c. per kg., intraperitoneal.		Depressed, vomiting, watering of eyes; <i>hemoglobinuria</i> . Improved and became quite lively. Killed on Aug. 4. Lived eight days.

1. *Clinical picture.*—Group I: These dogs received subcutaneous, intraperitoneal, and intravenous injections of immune serum, in varying amounts, averaging about 4 c.c. per kilogram. Although injections under the skin and into the peritoneum gave toxic results, still they were usually complicated by exudation and hemorrhage, and hence the intravenous method was most frequently used. During the injection the dogs became depressed, and shortly after began to vomit repeatedly; they emptied their bowels and bladder, lay about stupidly, or walked with a marked spastic gait in the hind legs. Then they either became rapidly worse, developing convulsions and coma, and dying in one-half to two hours, or recuperated somewhat, remaining sick, stupid, and listless for a few days, and then slowly improving. All the dogs had a marked hemoglobinuria, which appeared in about six hours and continued for several days. The feces were frequently dark and blood-stained. Shortly after the injection lachrymation

was noticed, and this continued for several days; frequently a conjunctivitis developed. A few hours after the injection a slight rise in temperature of one or two degrees was frequent. On the second or third day, at times some stiffness of the hind legs was noticed in walking, and Trousseau's phenomenon was easily produced. But it is not at all uncommon for normal dogs to show a similar spasm of the hind legs when irritated by pressure. No spontaneous convulsions, such as Mankovsky and Gontscharukov described on the third or fourth day, was noticed in these dogs. Some of the dogs lived four or five weeks, and during this time lost markedly in weight, and became cachetic and weak.

TABLE II.

Dogs Injected with Immune Goat Serum Previously Treated with Dog's Corpuscles.

Dog No.	Weight	Dose per Kilogram of Toxic Serum Treated with Blood Corpuscles of Dog	Reinjected	Results
6	9 kg.	Feb. 12, 1.8 c.c. per kg., intraperitoneal.	Feb. 19, 2.5 c.c. per kg., intraperitoneal.	Not much affected by first injection, but depressed and stupid for several days after second; <i>trace of hemoglobinuria</i> : watering of eyes; loss in weight. Killed on thirteenth day.
12	10 kg.	May 14, 3.5 c.c. per kg., intravenous.		Vomiting, frequent defecations and convulsions, and died in one hour.
16	7.5 kg.	May 26, 4 c.c. per kg., intravenous.	June 22, 4 c.c. per kg., intravenous.	Not much affected by first injection; marked depression after second, but rallied slightly, and then in a few hours convulsions and death in eight hours. Watering of eyes; <i>trace of hemoglobinuria</i> . Lived seven days after first injection.
18	4 kg.	June 11, 2 c.c. per kg., intravenous.	July 1, 1.5 c.c. per kg., intravenous. July 14, 2.5 c.c. per kg.	Slightly depressed after first injection and remained stupid and listless for several days; watering of eyes; <i>slight hemoglobinuria</i> ; loss in weight; gait staggering. After second injection, previous symptoms exaggerated; cachexia developed. After third injection, marked depression, and found dead next morning. Lived thirty-three days after first injection.
22	8 kg.	June 24, 2 c.c. per kg., intravenous.	July 1, 4 c.c. per kg., intravenous.	Vomiting and slight depression after first injection, watering of eyes, listless; <i>trace of hemoglobinuria</i> . After second injection, convulsions, and death in one hour. Lived seven days after first injection.
30	7.5 kg.	July 14, 3 c.c. per kg., intravenous.		Slight depression, watering of eyes, listless; <i>slight hemoglobinuria</i> ; constant loss in weight. Killed Aug. 20. Lived thirty-seven days.

Group II: These dogs received intraperitoneal and intravenous injections of immune serum, which had previously been digested with washed dog's corpuscles at 0° C. for thirty minutes. The dogs were not quite as sick as those in the previous group, but, although milder, the clinical picture resembled closely that of the former. A slight hemoglobinuria was produced.

TABLE III.

Dogs Injected with the Serum of Goats Treated with Colloid Matter.

Dog No.	Weight	Dose per Kilogram Colloid Matter Toxic Serum	Reinjected	Results
26	6.5 kg.	July 7, 6 c.c. per kg., intravenous.		Convulsions, and death in thirty minutes.
28	6 kg.	July 8, 2 c.c. per kg., intravenous.	July 13, 3 c.c. per kg., intravenous. Aug. 4, 5 c.c. per kg.	Vomiting, diarrhea, trembles all over, stupid and listless; recovered and became lively. Not much affected by subsequent injections; lost constantly in weight. Killed Aug. 15. Lived thirty-eight days after first injection.
32	7 kg.	July 28, 5 c.c. per kg., intravenous.	Aug. 4, 8 c.c. per kg., intraperitoneal.	Not very much affected; nausea; next day listless and stupid; some rigidity in hind legs; watering of eyes. Found dead next morning after second injection. Lived seven days after first injection.
33	8 kg.	July 29, 3 c.c. per kg., intravenous.	Aug. 8, 3 c.c. per kg., intravenous.	Depressed, listless for several days; collapse after second injection, but recovered. Killed Aug. 21. Lived twenty-three days after first injection.
34	9 kg.	Aug. 13, 4 c.c. per kg.		Not much affected; quite lively next day. Killed Aug. 21. Lived eight days after first injection.
35	7 kg.	Aug. 13, 7 c.c. per kg.		Vomiting, defecation, stupid and listless; but slowly improved. Killed Aug. 21. Lived eight days after first injection.

Group III: These dogs were injected intravenously with the serum of the goat, immunized with "colloid matter." A similar picture, but milder, was also observed in these animals. There was not, however, any demonstrable hemoglobinuria.

2. *Anatomical changes.*—Macroscopic: The post-mortem appearances varied with the mode of injection. The dogs receiving toxic serum subcutaneously showed in early cases a marked localized edema, and in those that survived several days a cyst-like accumulation of a bloody serous fluid was found. When the injections were given intraperitoneally, at first multiple subperitoneal

hemorrhages were found, but if the dog survived several days, a plastic peritonitis developed, in the exudate of which the colon bacillus was present. The conclusion naturally was drawn that the serum was endotheliotoxic, and permitted the colon bacillus to invade the peritoneum. The majority of the dogs received intravenous injections, and a rather constant picture presented itself after death. There was always a marked general pallor; flabby heart muscle; enlarged, bloody spleen; swollen, pale, fatty kidneys; and in the early stages the liver was swollen and chocolate-colored, in the latter yellow and fatty. The thyroid was frequently flabby, but otherwise it showed no changes.

Microscopic: Frozen sections, prepared from the various organs and stained with sudan III, showed fatty changes frequently in the thyroid and heart, and constantly in the liver and kidneys. Teased preparations revealed cloudy swellings in the same organs.

Thyroid gland, parathyroids, and hypophysis: The thyroid glands of normal and injected dogs were hardened in Flemming's fluid and were studied unstained for the fat-reacting granules described by Erdheim.⁴ The granules were found to be present and similar in all the glands.

The thyroids of the dogs injected with toxic serum show a decrease in colloid, which frequently is entirely absent and seems to have disappeared rapidly. The capillaries are often enlarged, and hemorrhages into the alveoli are occasionally observed. The epithelium presents a variable picture. Often the nuclei are swollen and vacuolated. In more marked cases, a partial or complete destruction of some of the cells and a marked desquamation of the remaining ones have occurred, so that the alveolus is distended with the dying cells and their fragments (see Plate IV, Figs. 1 and 2). In a number of the dogs dying later the epithelium has proliferated and filled the lumen with branched papillary-like processes, quite similar to those described by Halsted⁵ in his cases of compensatory hypertrophy following partial thyroidectomy (Plate V, Figs. 1 and 2). In no cases were there any recognizable changes in the parathyroid bodies. The same is

⁴ *Beitr. zur path. Anat. u. z. allg. Path.*, 1903, 33, 158-236.

⁵ *Johns Hopkins Hosp. Rep.*, 18(9), 1, 373.

also true of the hypophysis which was studied most carefully in serial sections by Dr. Le Count.

Liver: This shows marked active congestion in the dogs that succumbed in a short time. In those that survived a day or more the liver cells show various stages of degeneration, and several of the specimens present scattered areas of insular necrosis in which all semblance to cell structure is lost, the areas consisting of débris with skeletons of red blood corpuscles. Frequently large amounts of pigment are visible. Marked fatty changes are frequent.

Kidney: The kidneys show distinct changes. In animals that have lived a short time there is marked hyperemia, with occasional glomerular hemorrhages. The epithelium in those that survived a day or more is loaded with granules of various sizes and of a deep-brown color. In places these granules resemble fragments of red blood corpuscles. Where the cells appear more normal the lumen is filled with a dark-brown homogeneous mass, evidently a fusion of granules discharged from the living cells. In a fortunate section this change can be traced in a single tubule. The glomerulus is frequently crowded to one side and the capsule distended by a finely granular material. Acute degenerative changes, desquamation of the epithelial cells, and pronounced fatty changes are not uncommon.

Spleen, lymph glands, bone marrow: The sections of the spleen are usually loaded with red blood corpuscles in various stages of degeneration, and blood pigment is abundant. Numerous accumulations of red blood corpuscles, within a retaining membrane, are seen. The corpuscles are often fused into a brownish mass similar to that described in the kidney tubules, and others appear merely as shadows. Whether all these masses are phagocytic cells is hard to say definitely, as some appear as if composed of agglutinated red blood cells.

No marked changes were observed either in the lymph glands or bone marrow. The latter has not been studied as thoroughly as it ought to have been. In some of the glands are large phagocytic cells filled with disintegrating red corpuscles.

Pancreas, and adrenals: In neither of these were there any marked changes.

Nervous system: Dr. Rothstein examined the cords and brains of several dogs, with wholly negative results.

The changes in the dogs injected with "colloid matter immune serum" are not as marked as those described, but still they are similar. The most striking and constant feature is the marked or complete loss of colloid matter in the acini of the thyroid gland.

3. *Studies of the action of the immunized goat serum in vitro.*—Cytolysis and agglutination of thyroid cells: The technic described by Flexner and Noguchi⁶ in their work on the cytolysis of normal sera was closely followed. Normal goat serum causes rapid swelling and dissolution of the thyroid cells, and the conditions produced resemble those described in their work. The idea occurred that this swelling might be due to a hypotonicity of the serum, and by experiment it was found that the addition of 10 per cent. of a $\frac{n}{1}$ NaCl solution prevented the swelling of the thyroid cells and left them comparatively normal even after several hours. A further study, using the immune and normal serum previously modified in the above manner, showed that shortly after the mixture of serum and the thyroid emulsion an agglutination of the cells occurred with the normal as well as the immune serum, but decidedly more marked with the latter. In one-half to two hours the immune serum caused dissolution of many of the thyroid cells and left many free nuclei, whereas the normal serum only caused some clearing of the granules of the cells. Time did not suffice to carry out as large a number of experiments, modified and refined in various ways, as might be desired.

Specific precipitins: Experiments to test for specific precipitins were conducted with filtered and centrifugalized emulsions of thyroid tissue. Both the normal and immune goat serum caused slight precipitates if much serum was used, but with high dilutions (1:1,000) the immune serum caused a distinct cloudiness, while the normal produced only the faintest reaction or none at all.

Filtered solutions of colloid matter, in salt solution (0.85 per

⁶ *Jour. of Med. Research*, 1902, 9, 251-269.

cent.) or in magnesium sulphate solution (5 per cent.), when treated with the sera likewise gave distinct precipitates, especially marked in the case of the colloid matter immune serum, even in high dilutions (1:1,000). It is only by the use of high dilutions that anything like specific precipitation of filtered thyroid emulsion or of colloid matter is secured by the immune goat serum. To what extent the precipitate formed in such mixtures is dependent upon the presence in them of dog's blood-serum must be left undetermined for the present.

Hemolytic experiments: The serum of the immunized goats is strongly agglutinating and hemolytic for dog's corpuscles, 0.1 c.c. causing complete hemolysis of 0.1 c.c. of dog's blood. The question naturally arose whether this hemolytic property was due to the injection of dog's blood along with the thyroid emulsion. To determine this, the thyroids were first thoroughly washed with salt solution, as previously described, and another goat immunized; but it yielded a serum just as hemolytic as before. Again, the goat immunized with colloid matter likewise gave a hemolytic serum, though not quite as active. In the preparation of the colloid matter the thyroids were not previously freed from blood, and hence it remained possible that the blood might have been responsible for the hemolizing power of the goat serum. To test this a goat was immunized with extracts of dog's blood prepared exactly as the colloid matter. Increasing amounts of dog's blood were used—maximum 50 c.c.—and three injections of the extract given. The serum of the goat, however, did not gain in hemolytic power.

In their hemolytic action on dog's corpuscles the sera of the immunized goats present certain peculiarities that require further study before sufficiently well understood to merit full presentation. It is hoped that this may be accomplished soon. In the meantime it is interesting to find that immunization of goats with bloodless suspension of thyroid cells and with "colloid matter" of dogs causes a marked increase in the hemolytic power of the serum on dog's corpuscles.

That epithelial cells can cause the production of hemolytic substances has been shown by others. Most striking is the

experiment of v. Dungern,⁷ who, by injecting cow's milk, produced in the animal's serum substances not only cytolytic for epithelial cells, but also hemolytic. And the experiments of Pearce,⁸ with heteronephrotoxic and hepatotoxic serum, produced by the injection of washed, bloodless material, show these sera to have been hemolytic. Hence it seems that a strict cellular specificity cannot be claimed for cytotoxins. To what extent the changes observed in the other organs of my dogs were caused by the direct action of thyrotoxin or by the products of the hemolysis set up by the immune serum is another problem that demands further investigation.

SUMMARY AND CONCLUSIONS.

The chief results of the work presented may be summarized as follows:

The serum of goats injected with suspensions of the thyroid gland or with the thyroid colloid matter of dogs acquires many new and striking properties. Injected into dogs, it causes marked symptoms, prominent among which are depression, convulsions, vomiting, rapid breathing, hemoglobinuria, and early death in some cases, and in other animals that lived longer there were present also some fever, lachrymation, loss of weight, and progressive weakness. It cannot be claimed that there has been reproduced the exact picture presented by thyroidectomized dogs.

These clinical manifestations are associated with removal of colloid matter from the acini of the thyroid gland, desquamation and disintegration of the epithelial cells of the acini, followed in time by restitutive processes and the growth of papillary proliferations. The parathyroid bodies and the hypophysis show no changes. The liver, spleen, and kidneys present marked degenerative and pigmentary changes, which in large measure may be the result of the hemolytic properties of the serum injected, although it is possible that thyrotoxic serum contains cytotoxins for the cells of the liver and kidneys also.

In vitro, the thyrotoxic goat serum is more destructive and agglutinating for thyroid cells of the dog than normal goat serum.

⁷ *Munch. Med. Wchnschr.*, 1899, 46, No. 13, 405.

⁸ *Univ. of Penna. Med. Bull.*, 1903, 16, 217-235.

The toxic serum is markedly agglutinating and hemolytic for dog's corpuscles, even when obtained from goats injected with bloodless thyroid material and with colloid matter—an observation of great importance as regards the problem of community of receptors in various cells.

If it were possible to remove from the thyrotoxic serum the hemolytic as well as other direct and indirect cytotoxic actions referred to in the foregoing, it would seem to me warranted to expect that still stronger evidence could be obtained of a specific thyrotoxin. It may be, too, that the use of other animals might give less complicated results.

EXPLANATION OF PLATES IV, V.

FIG. 1.—Microphotograph of thyroid of dog No. 1 (see Table I). $\times 125$. Removal of colloid; desquamation and disintegration of epithelial cells.

FIG. 2.—Microphotograph of thyroid of dog No. 19 (see Table I). $\times 175$. Absence of colloid; beginning formation of papillary processes; cellular detritus in some of the acini; congestion.

FIG. 3.—Microphotograph of thyroid of dog No. 24 (see Table I). $\times 125$. Absence of colloid; papillary outgrowths; cellular stroma.

FIG. 4.—Microphotograph of thyroid of dog No. 6 (see Table II). $\times 125$. Same description as in Fig. 3. Blood corpuscles in acini.

A CONTRIBUTION TO THE CASUISTRY OF PLACENTAL AND CONGENITAL TUBERCULOSIS.*

MILIARY TUBERCULOSIS OF THE MOTHER IN SEVENTH MONTH OF PREGNANCY; TUBERCULOSIS OF PLACENTA; AGGLUTINATION THROMBI, CONTAINING MANY TUBERCLE BACILLI, IN PLACENTA AND FETUS.

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THE ancient problem, which so long busied clinicians and pathologists, as to the occurrence of a direct transmission of tuberculosis from the parent to the child, has, in so far as a maternal transmission is concerned, been settled beyond all doubt by a number of trustworthy observations based upon both anatomical and bacteriological findings. The frequency of such a transmission, however, must still be regarded as an open question. The dictum accepted by most writers at the present time, that the intra-uterine transmission of tuberculosis is possible, but extremely rare, needs the support of further research before it can be taken as final. It is true that but few undoubted cases of tuberculosis of placenta and fetus have been reported; but it is also true that no field of research has been so neglected as that of placental and fetal pathology. To what extent are systematic examinations of placentas made? In view of this neglect, it is not surprising that but few positive cases of placental and fetal tuberculosis have been observed; and some reason is afforded for the belief that those conditions are probably not so rare as they are at the present time supposed to be.

Further support is given to this belief by the fact that there is a constantly increasing number of cases reported in which an intra-uterine transmission of infectious diseases has been demonstrated beyond all doubt. Such a transmission from the mother to the fetus has been shown to occur in the case of small-pox, varicella, typhus fever, typhoid fever, relapsing fever, malaria, scarlatina,

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measles, anthrax, cholera, and infections with the pneumococcus and pus-cocci. While the number of such cases is on the whole not great, it is nevertheless increasing in proportion to the increasing frequency with which systematic examinations of the placenta and fetus are being carried out.

The greater frequency with which careful examinations have been made of the placenta and fetus of cattle and the large number of cases of bovine placental and congenital tuberculosis which have been reported strongly support the view that these conditions may not be so rare in man, and might be discovered more frequently were similar systematic observations made. The results of veterinary investigation, even though the latter be somewhat loosely carried out, favor the occurrence of placental transmission as a more frequent event than is generally believed.

As a result of these observations we have been obliged to modify somewhat our views concerning the rôle played by the placenta as a filter of micro-organisms contained in the maternal blood. At the present time the view is held by a preponderating majority of writers that the intact syncytial layer of the chorion forms a secure barrier against the passage of micro-organisms from the maternal to the fetal blood. There are several reasons, both clinical and pathological, for doubting the effectiveness and completeness of such protection. Before final dicta are accepted it will be necessary to enlarge greatly the casuistry of placental transmission; and it is desirable that large series of observations upon the fetus and placenta should be studied. The case described below is reported toward this end, as well as for its more immediate bearings upon the pathology of tuberculosis.

A number of writers have collected and analyzed the cases reported in the literature as congenital tuberculosis. Among the most important of such analyses are those of Gärtner,¹ Hauser,² D'Arrigo,³ and Cornet.⁴ A more or less complete bibliography is given by each one of these authors. It will be seen, however, from a survey of the cases analyzed by these writers that many

¹ *Zeit. für Hyg. u. Infektionskrankh.*, 1893, 13, 101-250.

² *Deutsches Arch. für klin. Med.*, 1898, 61, 221.

³ *Centralbl. f. Bakteriöl.*, 1900, 28, 683.

⁴ *Nothnagel Spec. Path. u. Ther.*, 1900, 14, 2, Abt. 2.

cases which have been regarded as undoubted examples of congenital tuberculosis have been diagnosed as such upon insufficient grounds, the evidence, both bacteriological and anatomical, being inadequate for the support of such a diagnosis. In but few cases is it possible to say positively that a placental transmission of tuberculosis had taken place, the other cases being doubtful because of the lack of bacteriological or anatomical evidence, the age of the child, possibility of extra-uterine infection, non-elimination of syphilis, etc. For this reason it will be of value to analyze again all such reported cases more closely than has previously been done, and to bring the bibliography up to the present date. In the analysis given below we have taken as criteria for the diagnosis of undoubted congenital tuberculosis the presence of characteristic anatomical changes and of tubercle bacilli, the development of the lesions within such a short time after birth as to preclude the possibility of extra-uterine infection, and the exclusion of syphilis. An analysis based upon such strict conditions has not yet been made, and our results, as given below, will be found to differ greatly from those of preceding writers. Many cases which have been regarded as undoubted examples of congenital tuberculosis we have put into the doubtful class, since they did not answer the above requirements so fully as to leave no room for doubt. Further, other cases reported as congenital tuberculosis we have thrown out altogether, since no sufficient proof was given of the nature of the process. The number of undoubted cases, therefore, becomes very small indeed; but a certain number of the cases placed in the doubtful list seem very probable instances of congenital infection, but are not reported with sufficient data to make the diagnosis undoubted.

I. CONGENITAL TUBERCULOSIS.

A. UNDOUBTED CASES.

(Diagnosis resting upon anatomical changes and the presence of tubercle bacilli.)

1891, SABOURAUD (*Soc. de Biologie*, Paris, Oct. 17, 1891).

Child aged eleven days, born of mother in advanced stage of pulmonary tuberculosis, who died shortly after delivery. Autopsy of infant showed presence of countless miliary tubercles in liver and spleen, in part showing caseation, and containing tubercle bacilli.

Woman forty years of age, suffering from advanced pulmonary tuberculosis, gave premature birth to a male child in the ninth month. The mother died two days after delivery. Autopsy showed chronic pulmonary tuberculosis, acute miliary tuberculosis of lungs, tuberculous meningitis. Placenta not examined. Child died twenty-four hours after birth. Autopsy showed miliary tuberculosis of lungs, liver, spleen, and kidneys. Tubercles also present in portal, mediastinal, bronchial, mesenteric, and retroperitoneal glands. The microscopical appearance was that of typical tubercles. Large numbers of tubercle bacilli found. No giant-cells present in the tubercles. Advanced stage of the process makes case undoubtedly congenital.

Child fifteen days old. Autopsy revealed typical caseous miliary nodules in spleen, liver, and lungs, containing numerous tubercle bacilli. These were also found free in the blood-vessels. Chronic tuberculous lesions were found in the liver. Mother was brought to hospital with pulmonary tuberculosis after birth of child. Case regarded as undoubtedly congenital, as such advanced lesions could not have formed during the short period of extra-uterine life.

New-born female child weighing 3,060 grams. Died of inanition after a few days. Nothing known of mother. Autopsy of infant showed general tuberculosis, most marked in liver. The spleen was somewhat enlarged, and contained so many tubercles that its surface presented a marbled appearance. Lungs contained a smaller number of tubercles. Brain and retina of both eyes free from tubercles. Large numbers of tubercle bacilli were found in the tubercles and also free in the capillaries. In some sections the bacilli were so numerous that when stained for tubercle bacilli the red areas were visible to the naked eye.

Mother in advanced stage of tuberculosis died three days after premature delivery in the seventh month. Placenta showed numerous caseous tubercles with tubercle bacilli. Inoculation of guinea pigs with portions of placenta gave positive results. Child died on twenty-eighth day. Autopsy showed miliary tubercles in lungs, liver, spleen, and endocardium of right heart. Typical tubercle bacilli present. Inoculation of rabbit with portions of fetal organs gave positive results. The tuberculous endocarditis is of especial interest as the first case noted in infants.

1894, LEHMANN (*Berl. klin. Wchnschr.*, 1894, 30, 646).

1894, HONL (*Bull. internat. de l'acad. de science de l'emper.*, F. J. I., Prague, 1894).

1896, USTINOW (*Arch. f. Kinderheilk.*, 1898, 25, 66).

1898, AUCHÉ ET CHAMBRELENTE (*Munch. med. Wchnschr.*, 1898, 45, 616).

B. PROBABLE OR DOUBTFUL CASES.

(Diagnosis resting upon anatomical appearances [gross or microscopical] only, without demonstration of presence of tubercle bacilli; or doubtful because of age of child, non-elimination of possible syphilis, extra-uterine infection, etc.)

1825, DUPUY (*Discus. de l'Acad. de Méd.*, 26 Apr., 1825). Dupuy found tuberculosis of the suprarenal in the sixth-month fetus of a phthisical mother. Anatomical evidence only. Doubtful case.

1825, HUSSON (*ibid.*). Found caseating and softened tubercles in the lung of a seven-month fetus of a woman suffering from advanced pulmonary tuberculosis. Evidence anatomical only. Case very doubtful.

1852, WEBER (*Beiträge z. path. Anat. des Neugebörn.*, 1852, 11, 64). Makes the statement that he had many times observed tuberculous cavities of the size of half a lobe in children under the age of three months. Very doubtful.

1861, JACOBI (*Compt. rend. du deuxième congrès de la tuberc.*, 1891, 327). (Not published until 1891.) Seven-month fetus of a mother suffering from chronic pulmonary tuberculosis had numerous caseating tubercles in liver, spleen, pleura, and peritoneum. Probable case. Anatomical evidence only.

1868, DEMME (*Bericht über die Thätigkeit des Jennerschen Kindersp. in Bern*, 1868, No. 6). Two cases. (1) Boy of five weeks. Sick from birth with fever and cough, showed on autopsy caseous nodules in both lungs and infiltration of bronchial and tracheal glands. Mother died of chronic pulmonary tuberculosis soon after delivery. (2) Child died on seventeenth day after delivery. Similar to first case. Tuberculosis in mother shown by physical signs. Both cases doubtful.

1873, CHARRIN (*Soc. des Sci. Méd. de Lyon*, 1873, No. 19). Seven-and-a-half month child of tuberculous mother, died three days after birth. Autopsy showed widespread tuberculosis of abdomen and abdominal organs. Scattered tubercles in lungs. Probable case. Anatomical evidence only.

1875, DEMME (*Bericht über die Thätigkeit des Jennerschen Kindersp. in Bern*, 1875, No. 13). Female child, twelve days old, of tuberculous mother. Autopsy showed caseous bronchial glands, and numerous caseating nodules in both lungs. In right apex and right lower lobe many cavities size of pea to a cherry. Mesenteric glands unchanged. Doubtful case. Anatomical evidence only.

1875, MERKEL (*Ohlendorff Zeitschr. f. klin. Med.*, 1884, 8, Heft 6). (Not reported until 1884 by Ohlendorff.) In January, 1875, patient developed pleuritis, followed by bronchial catarrh and infiltration of apices. In February she conceived. In June showed tuberculous laryngitis. By October patient had to be fed with tube. Child was born on November 4. On the 6th the mother died, autopsy showing tuberculous cavities and miliary tuberculosis. Child was very atrophic; was born with small yellow tumor on hard palate. On second day this discharged cheesy material; abscess then developed in left

greater trochanter. Child died of inanition. Autopsy showed caseous nodule in hard palate infiltrating the bone, caseation of cervical glands, caseous nodule behind left hip joint, not affecting joint. Probable case. Anatomical evidence only.

Female child, aged twenty-five days. Mother died of catarrhal pneumonia. Autopsy of child showed in the middle of the right cerebellar hemisphere a caseating tubercle of the size of a hazel nut. The microscopical examination showed the appearance of caseating tubercle. No tuberculosis elsewhere. Doubtful.

States that in autopsies of infants dying during the first months of life he had often found tuberculosis of such advanced stage as to make a congenital origin very probable. He refers also to cases of "wirklich congenitaler Tuberculose" reported by Ancell, Langstaff, Husson, Chaussier, Dupuy, Andral, Lobstein, Scanzoni, Rilliet and Barthez, Charrin and Demme. All of these cases doubtful.

Tuberculous mother, aged seventeen years. Child died on the ninth day after birth. Autopsy showed two small cavities, filled with caseous material, in posterior margin of lower right lobe. Microscopical examination confirmed the gross diagnosis of tuberculosis. Very probable case. Anatomical evidence. (Berti reports a second very doubtful case which may be entirely ruled out.)

Two cases: (1) Child dying on twenty-first day. Autopsy showed advanced tuberculous ulceration of intestine. (2) Child dying on twenty-ninth day of pulmonary tuberculosis. Both cases very doubtful. History meager. Evidence anatomical only. Extra-uterine infection not excluded.

Female child of tuberculous mother, dying five weeks after delivery. Child ill for three weeks with cough and attacks of vomiting. No evidence of syphilis. Died in eighth week. Autopsy showed advanced caseous tubercles of lung, liver, spleen, and kidneys. Bronchial and mesenteric glands enlarged, but not caseated. Intestines not ulcerated. One tracheal gland caseated. Probable.

Female child, aged eleven weeks, of tuberculous mother. Autopsy showed extensive cavity formation in right lower lobe. Doubtful. Extra-uterine infection not excluded.

Three-months-old child, with extensive caseation and cavity formation. Mother healthy; father tuberculous. Very doubtful. Extra-uterine infection not excluded.

1880, DEMME (*Bericht über die Thätigkeit des Jennerschen Kindersp. in Bern*, 1880, No. 17).

1882, BAUMGARTEN (*Folkmann's Samml. klin. Vortr.*, 1882, No. 218).

1882, BERTI (*Bolletino delle Scienze med. Bologna*, 1882; cited by GÄRTNER).

1884, DEMME (*Verh. d. 63 Vers. deutsch. Naturf. u. Aerzte*, zu Freiburg i. B., 1884).

1885, MONEY (*Brit. Med. Jour.*, 1885, 1, 1257).

1886, DEMME (*Bericht über d. Thätigkeit d. Jennerschen Kindersp. in Bern*, No. 24).

1886, QUEYRAT (*Contribution à l'étude de la tuberculose du premier âge*, Paris, 1886, 80 pp.).

- 1886, FLESCH (*Jahrbuch f. Kinderh.*, 1886, 25. Also *Pest. med.-chir. Presse*, Budapest, 1886, 22, 830). In eight cases out of five hundred autopsies on children in the early months of life tuberculous cavities (large or small) were found in lower lung lobes. Extra-uterine infection not excluded. Statement too inexact. Very doubtful. Evidence anatomical only.
- 1886, FROEBELIUS (*Jahrbuch. f. Kinderh.*, 1886, N. F. 24, 47-72). Found in 16,581 autopsies of children under two years of age 416 cases of tuberculosis. One died on third day, one in second week, one in third week, three at about three and one-half weeks, fourteen in second month, 119 in third month. No detailed account of these cases.
- 1886, HOUTINEL (*Thesis*, Paris, 1886). In 996 autopsies under second year, eighteen cases of tuberculosis; two dying in the first fourteen days after birth. No detailed account.
- 1887, LANNELONGUE (*Verneuil*, 1). Out of 1,005 cases of surgical tuberculosis in children from one to fifteen years of age examined within two years, four cases observed which he regards as of undoubted congenital origin: one child, six weeks old, with classical signs of tuberculosis of knee existing from birth; one, one month old, tuberculous osteoarthritis fourteen days after birth; one, three weeks old, tuberculous abscesses in left tarsus and right malleolar regions; one, sixteen days old, tuberculous otitis. Regarded as impossible for extra-uterine infection. (In another child of two months, right-sided tuberculous epididymitis with fistula. Scrotal engorgement some days after birth. Few details given concerning parents. Doubtful.)
- 1888, HUGUENIN (cited by LEBKÜCHNER, *Gaz. de Hôp.*, Paris, 1888, 61, 785-88). Two cases: one dying at the age of seven weeks of general tuberculosis; one of premature birth in the seventh month, with general tuberculosis and cavity formation in left apex. Doubtful. Evidence anatomical. No details.
- 1888, BOSSELUT (*Contribution à l'étude de la méningite tuberculeuse chez les jeunes enfants âgés de moins de deux ans*, Par., 1888). In a large number of autopsies on children dying of tuberculosis, meningitis found in one aged fourteen days, in two aged three weeks, in one aged six weeks, and in four aged eight weeks. Evidence not conclusive. No details.
- 1890, RINDFLEISCH (*Be-richt. d. 63. Naturforscherversammlung*, in Bremen, 1890). Child eight days old, of mother in advanced florid tuberculosis developing during pregnancy. Died of caseous pneumonia. General tuberculosis. Large caseous nodules in liver. Probable case. Anatomical evidence only.
- 1892, SARWEY (*Arch. f. Gyn.*, 1892, 43). Monster (cranioschisis) delayed delivery. Born in eleventh month. Mother apparently healthy. Father with cough and sputum. Child showed caseous and partly calcified nodules in upper cervical vertebrae. Guinea pigs inoculated. Three out of six developed tuberculosis after three months. Very doubtful. No tubercle bacilli at point of inoculation.

Stillborn monster (encephalocele). Caseation abscesses in three uppermost cervical vertebrae. No bacilli found. Evidence only anatomical. Details not given. Very doubtful.

(Observations of Parrot.) Child of eighteen days. Deep tuberculous ulcers in intestine. Caseation of tracheal and bronchial glands. Most probably congenital, as the extensive changes could hardly have occurred from extra-uterine infection.

Twenty-two other cases of tuberculosis in children under three months; one, four weeks old; one, five weeks (premature birth); two, six months; five, two months; eight, two and one-half months; five, two and three-quarter months. No details given. All doubtful cases. Purely clinical observations.

Child six weeks old with bronchial catarrh and otitis tuberculosa. Died four and one-half weeks later. Autopsy showed extensive tuberculosis of both lungs, diaphragm, liver, and kidneys, with advanced caseation. Wassermann believed case to be acquired from the relative of mother with whom the latter and child had resided for a short time when child was ten days old. Correctness of this opinion questioned by Baumgarten and Lebküchner. Very doubtful case.

Reports three cases, aged thirty-one days, thirty-eight days and sixteen weeks respectively, of combined tuberculosis and syphilis. Mother in first and third cases tuberculous. Autopsies of children showed advanced tuberculosis in all three cases. Tubercle bacilli present. Age of children and the fact that congenital syphilis predisposes to rapid development of tuberculosis make these cases doubtful.

Child three weeks old showed caseous tubercles in lung, spleen, bronchial, and mesenteric glands. Doubtful case. Full details not given.

Out of one thousand autopsies on children there was observed three times, in children of four, five, and six weeks, an intense tuberculosis of bronchial glands, which he regarded as congenital. No details given. Doubtful.

Mentions one case, dying on the twentieth day, of premature birth. Mother, suffering from advanced tuberculosis, died shortly after the child. On autopsy child found to have caseous bronchial glands, and miliary tuberculosis in lungs, none in liver, alimentary tract, or spleen. Regarded as probably congenital from advanced nature of lesions. Doubtful.

1892, BAUMGARTEN U. ROLOFF (*Arbeiten aus dem path.-anat. Institut zu Tübingen*, 1892).

1892, LEROUX (*La tuberculose du premier âge, d'après les observations inédites du prof. Parrot, Verneuil*, Paris, 1888).

1894, WASSERMANN (*Zeitschr. f. Hyg. u. Infektionskrankh.*, 1894, 17, 343-54).

1894, HOCHSINGER (*Wien. med. Bl.*, 1894, 17, 255-57).

1895, STRAUS ("La tuberculose et son bacille," *Rev. gén. de l'antisept. méd. et chir.*, 1895, 8, 97).

1896, KISSEL (*Arch. f. Kinderheilk.*, 1898, 25, 67).

1896, HOLT (*Med. News*, 1896, 69, 656-59).

1897, HENOCH (*Vorles. über Kinderkrankh.*, Berlin, 1897). Father died of tuberculosis. Child ill from sixth week with multiple tuberculous abscesses over entire body. Died in fourth month of inanition. Autopsy showed advanced pulmonary tuberculosis, intestinal tuberculosis, and caseation of lymph glands. Very doubtful. Extra-uterine infection not excluded.

1898, BONNET (*Lyon. Méd.*, 1898, 87, 224). Mother died of pulmonary tuberculosis two months after delivery. Male child, ill from birth, died at three months. Autopsy showed both lungs studded with caseous tubercles, tuberculous ulcers in ileum, caseous tuberculosis in mesenteric glands, kidneys, spleen, and liver. Fatty liver. Stage of lesions, and the fact of illness from birth, the child having been kept from danger of infection, given as reasons for regarding case probable.

1899, JOHNSON (*Phil. Med. J.*, 1899, 3, 231). White female child born of mother with tuberculosis of bladder. At birth infant very weak, small: could not nurse. Emaciation increased. Made efforts at coughing. Died during a profuse pulmonary hemorrhage. Father healthy. Urine of mother contained blood, pus, and tubercle bacilli. Placenta probably tuberculous (see below). Autopsy of child showed extensive tuberculosis of right lung, pleura, and pericardium; miliary tuberculosis of left lung. Tubercle bacilli could not be found in fetal organs. Evidence not complete, but most probably a case of congenital tuberculosis.

1899, F. LEBRÜCHNER (*Arbeit. aus dem path.-anat. Institut zu Tübingen*, 1899, 3). Two cases: (1) Mother of tuberculous family, suspicious symptoms, but case not certain. Child short of breath and coughed from birth. On section advanced tuberculosis in all organs. (2) Case similar, but child older. The first case may be regarded as probable, though evidence is incomplete.

1900, FRIEDMANN (*Deutsch. med. Wchnschr.*, 1900, 26). Child twenty-six days old, with solitary tubercle in right apex. Not conclusive.

1900, LYLE (*Phil. Med. J.*, 1900, 5, 219). Negress, twenty-two years of age, in seventh month of pregnancy, suffering from chronic pulmonary tuberculosis. Died two days after premature delivery. Autopsy showed chronic tuberculosis of both lungs and intestinal ulceration. Placenta appeared normal. Child apparently full-term; weighed about three pounds. Ill from birth; subnormal temperature for four weeks, followed by fever; died in eighteenth week. Autopsy revealed extensive tuberculosis of lungs, liver, spleen, and kidneys; tubercle bacilli present in the caseous areas. The great number of tubercles of same advanced stage, the fact that child was ill from birth and had been kept under such conditions as to exclude possibility of infection, are the reasons adduced for considering this case as congenital. It is doubtful, however, because of the long period (nineteen weeks) between birth and death.

II. PLACENTAL TUBERCULOSIS.

A. UNDOUBTED CASES.

(Diagnosis resting upon demonstration of both histological changes and the presence of tubercle bacilli in the placenta.)

CASE I.—Woman, aged twenty-six years, died of general miliary tuberculosis in seventh or eighth month of pregnancy. Male child removed by Caesarean section five minutes after death of mother; showed no signs of life. Well-developed. The only abnormal changes found were two grayish nodules in the right apex. Pieces of liver and spleen were inoculated into guinea pigs. General appearance of placenta was normal. In several places small miliary nodules, grayish, semi-translucent, and sharply outlined, were found. The microscopical examination of fetal liver and lungs gave no appearance of tuberculosis. No tubercle bacilli found. The placental nodules presented the appearance of typical caseating tubercles, containing few tubercle bacilli.

1893-94, LEHMANN
(*Deutsch. med. Wchnschr.*, 1893, 19, 200-202).

CASE II.—Mother died of chronic tuberculosis of lungs and larynx. Child died ten days after birth. Presented no evidences of tuberculosis at autopsy. Typical caseating tubercles containing tubercle bacilli found in the placenta.

(*Berl. klin. Wchnschr.*, 1894, 31, 601).

CASE I.—Woman, aged twenty-six, in seventh or eighth month of pregnancy, died of chronic pulmonary tuberculosis and miliary tuberculosis. Child removed by Caesarean section; lived two hours. No histological changes of tuberculosis or tubercle bacilli found in fetus. Placenta appeared normal to the naked eye. Numerous tubercle bacilli found in smears from placenta. Animal inoculations negative. On microscopical examination placenta showed typical tubercles in all stages. Tubercle bacilli in large numbers.

1894, SCHMORL-KOCKEL
(*Ziegler's Beiträge*, 16 313).

CASE II.—Mother, aged twenty-five, died of general miliary tuberculosis. Fetus removed at autopsy. No evidences of tuberculosis in fetal organs. Tubercle bacilli found in fetal liver. Periportal tissue and lymph glands without anatomical changes. Animal inoculations negative. Typical tubercles containing tubercle bacilli found in placenta.

CASE III.—Woman, aged thirty-three, died in ninth month of pregnancy of chronic pulmonary tuberculosis. Child removed by Caesarean section; dead when uterus was opened. Male child, showing no changes of tuberculosis. No tubercle bacilli found in fetal tissues. Placenta presented no naked-eye appearances of tuberculosis. Animal inoculations with fetal tissue negative.

Microscopically the placenta showed typical tubercles in varying stages, not so numerous as in Cases I and II. Tubercle bacilli present in large numbers.

1896, WARTHIN, (*Med. News*, 1896, 69, 319-24.) Case of tubal gestation with tuberculosis of tubes, placenta, and fetus. Rupture of tubal sac in fourth month; operation. Advanced tuberculosis of tubes, and wall of sac. Chorionic villi involved directly by extension from wall of tube. Typical tubercles in chorionic villi. Few tubercle bacilli found.

1898, AUCHÉ AND CHAM-BRELENTE (*Münch. med. Wchnschr.*, 45, 1898, 616). Mother, far advanced in tuberculosis, died three days after premature delivery in seventh month. Placenta showed numerous caseous tubercles containing tubercle bacilli. Inoculation of guinea pig positive. Child lived twenty-six days. Autopsy showed extensive tuberculosis.

1899, WARTHIN ("Unusual Localizations of Tuberculosis," *Medical News*, 1899). In one of fifty successive placentas examined at the Stadt-Krankenhaus in Dresden (Schmorl's Laboratory) miliary tubercles in all stages were found in full-term placenta. Contained few bacilli. Nothing known of mother or child, as placenta could not be identified.

B. PROBABLE CASE.

1899, JOHNSON (*Phil. Med. J.*, 1899, 4, 930). Mother had tuberculosis of bladder. Child lived a little over three months. Ill from birth. Autopsy showed extensive and old tuberculous lesions. Placenta contained necrotic areas. On microscopical examination areas of necrosis, diffuse inflammation, and foci of coagulation necrosis were found. No giant-cells and no tubercle bacilli. The character of the changes, as shown by the figure given, makes it probable that the placental lesions were tuberculous.

III. TUBERCLE BACILLI IN FETUS AND PLACENTA WITHOUT HISTOLOGICAL CHANGES.

A. UNDOUBTED CASES.

(Diagnosis resting upon the demonstration of the bacilli by staining or by inoculation of animals.)

1883, LANDOUZY AND MARTIN (*Rev. de Méd.*, 1883, 3). A portion of the lung of the six and one-half months' fetus of a tuberculous mother, dying a few days after delivery, was inoculated into the peritoneal cavity of guinea pigs. Animals died of general tuberculosis four months afterward.

1885, KARTH AND CHARIN (*ibid.*, 1885, 5, 659-72, cited by FRIEDMANN). Guinea pig inoculated with portion of placenta from tuberculous mother. Result positive.

1891, HERRGOTT AND HAUSHALTER (*Ann. de Gyn. et d'Obst.*, 1891, 36, 1, 100). Woman dying of chronic pulmonary tuberculosis in sixth month of pregnancy. Inoculation of guinea pigs with amniotic fluid positive. The only case of the kind recorded.

Seven-month fetus extracted by Caesarean section from mother, twenty-three years of age, dying of miliary tuberculosis. Tubercle bacilli found in fetal liver, in intervillous spaces, and in chorionic villi. No histological changes of tuberculosis found. Inoculation of guinea pigs with portions of fetal liver were positive in three cases.

Two cases: (1) The seven-month fetus of a tuberculous mother showed no histological changes. Inoculation of guinea pig with portions of fetal organs gave positive results. (2) The five-month fetus of mother dying of tuberculosis presented no histological changes. Placenta apparently normal. Inoculations with portions of placenta and heart-blood of fetus were positive; inoculations with fetal liver doubtful; inoculations with portions of lung and brain were negative.

The seven-month fetus of mother dying of acute miliary tuberculosis showed no histological changes. Bacilli in fetal blood. Inoculation of guinea pig with portions of placenta and fetus gave positive results. Case like the one of Schmorl and Birch-Hirschfeld.

Mother died of pulmonary and intestinal tuberculosis fourteen days after delivery. Child died on fourth day. Numerous tubercle bacilli were found in fetal liver, spleen, and kidneys. No histological changes found. Inoculations of guinea pigs with blood from umbilical cord positive.

Two cases: (1) Mother died of acute miliary tuberculosis eight days after abortion. No tuberculous changes or tubercle bacilli found in fetus. Inoculations of guinea pigs with portions of liver and placenta, and with fetal blood, were positive. (2) Mother with advanced tuberculosis. Child died ten days after birth. No tuberculous changes or tubercle bacilli found in fetus. Inoculations with venous blood, portions of fetal organs, and placenta were positive.

Three cases of abortion in mothers suffering from general tuberculosis. No histological changes found in fetus in any one of the cases. In only one case tubercle bacilli were found in the fetal liver, periportal tissue, and lymph glands. The placenta in each case contained bacilli and tubercles. The inoculation with portions of fetal organs was negative in all three cases.

Portions of the placentas and fetal organs from six mothers suffering with chronic pulmonary tuberculosis were inoculated into animals, with positive results in four cases. In one case tubercle bacilli were found in great numbers in the fetal organs.

1891, SCHMORL AND BIRCH-HIRSCHFELD (*Beiträg. z. path. Anal. u. z. allg. Path.*, 1891, 9, 428-39).

1891, LANDOUZY (*Rev. de Méd.*, 1891, 11, 411, 431).

1892, AVIRAGNET (*Union Méd.*, 1892).

1893, THIERCELIN AND LONDE (*Méd. mod.*, 1893, 4, 398).

1893, LONDE (*Rev. de la Tuberc.*, 1893, 125-43).

1894, SCHMORL AND KOCKEL (*Ziegler Beiträge*, 1894, 16).

1895, LONDE (*Compt. rendus Acad. de Sci.*, 1895, 121, 544).

- 1896, BUGGE (*Ziegler Beiträge*, 1896, 19). Eight-month fetus of mother with miliary tuberculosis lived but thirty hours after birth. Mother died shortly afterward. Tubercle bacilli were found in the blood from the umbilical vein and in the liver vessels. No histological changes found. Three guinea pigs inoculated with blood from the umbilical vein. Portions of fetal liver and lung gave positive results.
- 1896, DOLÉRIIS AND BOURGES (*Semaine méd.*, 1896, 375). Mother died of acute miliary tuberculosis three weeks after delivery of a seven-month child. The marasmic infant died five weeks after birth. No tubercle bacilli or histological changes of tuberculosis found in child. The inoculation of a guinea pig with the heart-blood of the child gave positive result of special interest, in that the child should contain bacilli for five weeks without occurrence of tissue changes. Case doubted by Hauser and Cornet for this reason.

B. DOUBTFUL CASES.

(Regarded as doubtful because of meagerness of details, length of time elapsing after inoculation, lack of control experiments, etc.)

- 1883, LANDOUZY AND MARTIN (*Rev. de méd.*, 1883). Mother died of tuberculosis in fifth month of pregnancy. Portions of the placenta and twenty to twenty-five drops of fetal heart's blood inoculated into three guinea pigs. All died of general tuberculosis four months afterward. Three guinea pigs inoculated with portions of fetal liver, lung, and brain remained alive.
- 1890, ARMANNI (*X. Internat. Med. Cong. in Berlin*, 1890). Mother died of tuberculosis in seventh or eighth month of pregnancy. Fetus showed no histological changes. Portions of spleen, liver, and brain were inoculated into two guinea pigs. One died four months afterward of general tuberculosis; the other not affected. Secondary infection of pig not excluded.
- 1893, THIERCELIN AND LONDE (*Méd. mod.*, 1893, 4, 398). Mother died fourteen days after delivery. Had pulmonary and intestinal tuberculosis. Child died a few days after birth. No autopsy. Portion of placenta placed in peritoneal cavity of guinea pig gave positive result. Actual condition of child not known.
- 1893, LONDE (*Rev. de la tuberc.*, 1893, 125-43). Mother died of tuberculosis ten days after delivery. Age of child not given. No autopsy. Inoculation of guinea pig with portion of placenta positive.
- 1895, BAR AND RENÓN, (*Annal. de Gyn. et d'Obst.*, 1895, 41, 43, 44; see also *Sem. méd.*, 1895). Five cases of delivery of tuberculous mothers. The blood of umbilical vein was injected immediately into guinea pigs. Three cases gave negative results; two were positive. Of the latter one case showed no apparent lesions in placenta or fetus. No bacilli found. Mother had late stage of pulmonary tuberculosis. Three animals were inoculated with pieces of liver and

lung and with peritoneal fluid. The one inoculated with portion of liver had local lesion and general tuberculosis; the one inoculated with peritoneal fluid had tuberculosis of spleen, but no local lesion.

The second case is very doubtful, secondary infection not being excluded. Mother had tuberculous cavities in lungs. Child died of bronchopneumonia on the fortieth day after birth. Placenta appeared normal. Two guinea pigs were inoculated with blood from umbilical vein; one negative; the other developed local and general tuberculosis.

The placentas of thirteen tuberculous mothers, and in a few cases portions of the fetus also, were examined for tubercle bacilli. These were found once in the maternal blood. In eight cases in which the fetus was examined histologically and animals inoculated, no histological changes were found. In only one case the inoculation with placenta was positive. Report inexact and contradictory.

1895, BOLOGNESI (Thesis, Paris, 1895.)

Mother had chronic tuberculosis. Child died four days after delivery. Autopsy showed pneumonia with fresh fibrinous pleuritis. No microscopic changes of tuberculosis. Portions of an apparently healthy bronchial gland were inoculated into guinea pig. Pig showed general tuberculosis on the thirty-seventh day. Neither histological changes nor tubercle bacilli could be found in serial sections of another gland. Author excludes accidental infection of inoculated animal and regards case as a typical tuberculosis inoculation. No control of experiment. Secondary infection of wound not excluded.

1896, HENKE (*Arbeit aus dem path.-anat. Institut. z. Tübingen*, 1896, 2.)

A number of investigators have also examined the fetus and placenta of tuberculous mothers for tubercle bacilli and evidences of tuberculosis, but without positive results. Leyden, Wolff, Houtinel, Vignal, Londe, Grancher and Straus, Bar and Renón, Bugge, Borst, Heinemann, and others have examined fetal and placental tissues for tubercle bacilli, by staining and inoculation of animals, with negative results. On the other hand, such investigation has been carried out in cattle by a large number of veterinarians with positive results. To these (Johne, 1885) we owe the first positive proof of the intra-uterine transmission of tubercle bacilli.

The above synopsis of the literature shows, therefore, a paucity

of thoroughly studied cases bearing upon the important question of human placental and congenital tuberculosis. The completeness of the study which we have been able to give one case makes it of especial value at this time.

HISTORY OF CASE.

Mrs. H. M., aged twenty-seven, was seen in consultation with Dr. Neil Gates, of Dexter, Mich., Oct. 17, 1901.

The patient complained of frequent and painful micturition, great weakness, sweating, and cough.

Father, brother, and three aunts died with pulmonary tuberculosis.

Patient has been married between three and four years. Has one child living and in apparent perfect health. She has always done housework. She was born in Colorado, and was always sickly as a child. At the age of two weeks she had "a severe cold in the head, stopping of the nose, and croupy cough." These symptoms lasted for about one week, and were followed by "breaking and discharge" from both ears. Soon after the beginning of the ear symptoms she was brought from Colorado to Michigan, when her nose began to discharge a thick, yellowish, creamy substance in abundance, having an offensive odor and similar to the discharge from the ears. The left ear discharged more than the right. The hearing has been impaired ever since. An out-of-door life resulted in a moderately quick recovery from the acute symptoms. A chronic discharge from both ears, however, persisted.

Except that she was troubled with a frequent desire to urinate, which was a noticeable symptom, she was well, but frail, for the remainder of her childhood.

Patient began to menstruate at the age of fourteen, with little or no pain. For a year or more prior to present pregnancy, menses have been irregular, at one time skipping two months. The flow was profuse, at times lasting six or seven days. No miscarriages.

Seven years ago micturition began to be more frequent, producing a burning sensation, and at times causing actual pain. She sought relief in many ways, but was unsuccessful. After her marriage, a little over three years ago, these symptoms grew worse, and since that time it has been necessary for her to urinate every half-hour, sometimes every fifteen minutes. This was especially noticeable if she rode in a carriage. When going to town, a thirty-minute drive, she was obliged to get out of the carriage two and sometimes three times. The amount of urine at each micturition was small, and toward the last amounted to only 3 or 4 c.c. For the last six months she has urinated every half-hour through the night. At no time did these symptoms abate. They grew steadily worse. Neither her husband nor her mother ever noticed any blood in patient's urine excepting during menstruation. She never complained of her feet or her legs swelling, nor was this symptom ever noticed by her husband, mother, or her physician, who saw her frequently for three years.

She had a chronic cough for a year or two, expectoration always being

scant. During the past summer her cough was dry and distressing, and was unrelieved by taking medicine. She has had attacks of sweating until present illness began.

Present illness.—On Thursday, October 3, 1901, patient went to a county fair. The day was cold. She was dressed in light summer clothes. When she reached home, after a drive of four miles, she complained of being very cold. Her lips were purple. The next day she began to cough very hard. On Sunday, October 6, she had a fever, and Dr. Gates was summoned. Her temperature, at this time, "was 102° F., and her pulse weak and rapid." She was put to bed, and soon after this she had a distinct chill: her temperature rose higher, and she complained of her throat being sore. From this time until the patient was seen by Dr. Cowie her temperature ranged from normal to 100° F. in the morning, to from 101° to 103° in the evening.

She was first seen by Dr. Cowie on the afternoon of October 17, 1901, two weeks after she was taken ill. She looked very weak and tired. She was sweating profusely all over, more markedly on the forehead. Her eyes were bright, her cheeks flushed, and her respiration quick and shallow. The appearance of her face was like that of croupous pneumonia. Her speech was slow. She suffered much pain from micturition, which now occurred every five or ten minutes. Her temperature was 101°, pulse 110 and fairly strong, respiration about 40. She was in about the fifth (?) month of pregnancy.

PHYSICAL EXAMINATION.

(October 17, 1901.)

The patient is emaciated: musculature is small and wasted. All of the ribs are visible. The skin is slightly sallow, except over the face where it is flushed. The panniculus is very thin. There are lineae albicantiae over the lower part of the abdomen, and a distinct linea fusca below the umbilicus. There is no edema. The hearing is impaired in both ears. She has a dry cough, but no expectoration. Lies in active dorsal position.

The thorax is long; the epigastric angle is narrow. The breathing is quick, shallow, and labored, the accessory muscles being brought into use. The expansion is slight.

Palpation.—The vocal fremitus is increased on both sides, particularly over the upper left lobe.

Percussion.—There is impaired resonance over the upper right lobe, while the percussion over the corresponding area on the left elicits a dulness having in it a slight resonant quality. The note is distinctly higher and its quality duller than that obtained on the right side. Over the back the area of impaired resonance is more extensive. On the left it extends to the lower border of the scapula; on the right, over the upper third of the scapula; below this line the resonance approaches more closely that of a normal lung. Apices and lower lung borders were not noted at the time.

Ausculation.—Inspiration is short and jerky; expiration is short, but is slightly longer than inspiration and is followed by a short pause. Over the left upper lobe the breathing is blowing, and a number of fine crackling subcrepitant râles are heard, occasionally a dry piping râle. No moist râles.

Over the upper right lobe there is increased vesicular breathing. Blowing breathing is heard on this side only over a small area two fingers to the right of the sternum in the second intercostal space. There are no râles below or above the clavicle, except an occasional crackling one. No friction sounds are heard.

The examination of the heart is negative.

Abdomen.—The abdomen is markedly above the level of ribs. Pregnancy about fifth month (?). Fetal movements and heart-beat present. Further examination unsatisfactory.

No sputum could be obtained. Patient was too weak to expectorate and swallowed the little she raised. No microscopic examination was made, because no sputum could be obtained. Her sputum had never been examined for tubercle bacilli.

Urine was obtained per catheter under sterile conditions in a sterile bottle, but by mistake the bottle was thrown out by one of the family. Large numbers of tubercle bacilli were found in the urine obtained from the bladder at autopsy (see autopsy record). (During last two years her urine had been examined chemically a number of times; albumin was a constant constituent. No microscopic examination was made.)

A diagnosis of acute miliary tuberculosis was given as the result of the consultation.

Dr. Cowie did not see the patient again until Monday, October 21, when the case appeared to be approaching rapidly a fatal issue. The fetal movements and heart-beat were absent. Relief from painful micturition was secured by the use of a sterilized soft rubber catheter retained in position.

As was expected, she aborted early on the following morning. The fetus gave no evidence of life. The fetus and membranes, placenta and cord, were taken to the pathological laboratory almost immediately after the abortion. The patient died on the same day, about twelve hours later. Through the efforts of Dr. Gates an autopsy was secured. This was performed by Dr. Warthin, at 11 A.M., October 23, 1901, at patient's home in the country. Measurements and weights could not under the circumstances be obtained.

AUTOPSY PROTOCOL.

Cadaver of slender build. No anomalies or deformities. Abdomen greatly distended. Muscles small, greatly emaciated. Rigor mortis present throughout. Very small amount of panniculus. Slight edema over ankles. Body heat absent. Skin slightly sallow; faint linea fusca below the umbilicus. Very slightly marked linea albicantiae over the lower portion of abdomen. Moderate hypostasis over back. Post-mortem percussion gave dullness over both lungs, more marked over the left.

Brain and spinal cord were not examined.

No fluid in peritoneal cavity; peritoneal surfaces dry and sticky. Transverse colon enormously distended, lying in a downward curve extending below the level of the umbilicus. Ascending colon slightly distended; the descending also, as far as the sigmoid, is greatly distended. Pelvis filled with the enlarged and contracted uterus. Otherwise the position of abdominal organs is negative.

Diaphragm at fourth intercostal space on right, fifth rib on left.

Mammæ small; areolæ pale. A small amount of colostrum can be expressed from the nipples. On section the acini are hyperplastic, the ducts enlarged and filled with a yellowish viscid fluid.

Examination of *mediastinum* negative. No remains of *thymus*. The *lungs* are voluminous, completely covering the small heart. Apex in fourth intercostal space just outside the left parasternal line. No fluid in pleural cavities. Pleural surfaces cloudy. No adhesion on right; over the apex and base of the left lung there are old firm adhesions.

Pericardial fluid slightly increased and slightly cloudy; subepicardial fat somewhat increased. Parietal pericardium negative; a few remains of old adhesions over the visceral layer. Heart small, size of cadaver's right fist. Ventricles filled with cruor. Right auricle contains large, white, ante-mortem jelly clot. Walls of normal thickness; fat slightly increased. Heart muscle firm. Valvular orifices and flaps negative. No tubercles in heart or pericardium.

The *left lung* is moderately voluminous, firm and inelastic, especially over the lower lobe, where it is almost airless. The pleura of lower lobe is cloudy and covered with small pin-point nodules which on section are firm, semi-translucent, and showing caseous centers. The cut surface is bluish-red, bleeds freely; it is studded with countless small, grayish, pin-point nodules, some of the largest showing caseation.

The *right lung* is voluminous, of much lighter color, particularly the upper and middle lobes, which are pink, the lower one red. Beneath the pleura there are everywhere small pin-point nodules, somewhat smaller than those of the left lung. Beneath the pleura on the anterior surface of the lower lobe there are several flattened tubercles size of a pea and deeply pigmented. On section the centers show grayish caseation. The section of the right lung is similar to that of the left, showing countless miliary tubercles about the size of a pin-head, very few being caseous.

Ante-mortem white clots are present in pulmonary veins; red clots in the arteries. The *bronchi* contain a large amount of frothy sputum; their mucosa is deeply injected. The *bronchial glands* are enlarged; softened, moderately anthracosed, and contain a few miliary tubercles. Careful examination of the *thoracic duct* shows no involvement by the tuberculous process. The examination of *esophagus* and *thoracic vessels* is also negative.

The examination of *nose*, *tonsils*, *pharynx*, *larynx*, *trachea*, *cervical vessels*, and *nerves* is negative. The thyroid is slightly enlarged, on section negative. Parathyroids negative. No tuberculous process found in *cervical lymph glands*.

The *spleen* is a little over twice as large as normal; its capsule slightly thickened. Consistency very soft. Organ is flattened. There are two shallow notches on the anterior surface, and one deep one on the posterior edge. On section the cut surface is deep bluish-red. Scattered throughout the pulp are numerous miliary nodules, hard and semitranslucent, none showing caseation.

The examination of the *adrenals* is negative. No tuberculosis.

The *left kidney* is enlarged. The fatty capsule is rich in fat; the fibrous

capsule is adherent. On section the medullary pyramids are found to be almost entirely destroyed, and replaced by tuberculous abscesses showing advanced caseation and liquefaction. The abscess cavities are filled partly with caseous material and partly with a thin cloudy fluid. They are encapsulated, the capsule consisting of a dense, firm, hyaline layer of connective tissue. The pelvis is greatly dilated; its mucosa thickened and ulcerated, presenting numerous caseating nodular masses. The cortex is very pale, semitranslucent, and firm. The outlines between labyrinth and medullary rays almost entirely lost. Miliary tubercles are scattered throughout the cortex.

The *right kidney* is much smaller than the left. There are encapsulated tuberculous abscesses in the pyramids. Otherwise the appearance is similar to that of the left.

The *ureters* are dilated, their mucosae thickened. They contain cloudy urine and caseous material. The *bladder* contains two tablespoonfuls of thick cloudy fluid. The mucosa is thickened, covered with fibrin flakes and cheesy material, and presenting small erosions or ulcerations.

The examination of the *stomach*, *large* and *small intestines*, *appendix*, and *rectum* is negative, with the exception of the distention noted above.

The *liver* is much enlarged; capsule negative. On section the cut surface shows a moderate nutmeg appearance. No tubercles visible to the naked eye. The *gall-bladder* contains a small amount of bile; no stones are present. The examination of the *portal vein* is negative.

The *external labia* are edematous and purplish-red in color. The vagina is dilated; its mucosa deeply congested and covered with a bloody discharge. The examination of the urethra is negative. The uterus is the size of a large cocoanut: presents the appearance of a post-partum organ. It is firmly contracted and lies deep in the pelvis. Cervix is dilated; no laceration. Uterine vessels greatly congested. *Placental site* in upper right anterior portion of fundus. Some placental tissue *in situ* covered with large blood-clots. Entire endometrium covered with decidual shreds and large clots. On section the decidua at the placental site shows thrombosed sinuses. No evidences of tuberculous processes visible to naked eye.

No pathological changes in the *tubes* or *ovaries* apparent to the naked eye. True corpus luteum in left ovary. The blood-vessels of the broad ligament are enormously congested.

The examination of *bones*, *joints*, and other *peripheral structures* is negative.

Portions of all organs were fixed in formalin, mercuric chloride, and alcohol; imbedded in paraffin, and stained with ordinary stains. Specific stains for tubercle bacilli (carbol fuchsin) and fibrin (Weigert's) were also employed.

MICROSCOPICAL.

Lungs. Sections taken from different portions of both lungs show a general miliary tuberculosis of both organs. The majority of the tubercles are in a stage of advanced caseation, many of them showing much formation of reticulum and beginning encapsulation. Giant cells are present in large numbers in the majority. In many of the larger branches of both pulmonary

arteries and veins miliary tubercles are found projecting into the lumen of the vessel and apparently arising from the wall of the vessel (hematogenous metastasis). Tubercle bacilli are found in abundance in the caseating nodules. The advanced caseation, the abundant reticulum, and the attempt at encapsulation seen in many of the tubercles justify the diagnosis of subacute miliary tuberculosis. The sections show further a marked congestion, edema, and extensive pneumonia.

Bronchial glands.—Scattered miliary tubercles of same stage of development as those in the lungs.

Heart muscle.—Slight atrophy and fatty degeneration. Slight fatty infiltration beneath epicardium. No tubercles found in heart.

Spleen.—Numerous miliary tubercles scattered throughout pulp, of same stage of development as those in lungs. Marked congestion. Coagulation necrosis of some of the follicles.

Adrenals.—Negative, post-mortem change of medullary portion.

Kidneys.—The large abscesses in the medullary pyramids are tuberculous: the abscess cavity filled with caseating material: the wall of the abscess composed of epithelioid tissue containing numerous giant cells. There is also a distinct capsule formation about the largest abscesses. The medullary structure between the tubercles is for the greater part destroyed. The cortical portion presents the appearance of a primary interstitial nephritis. Scattered throughout the cortex are numerous miliary tubercles of various stages of development. The pelvis shows an advanced stage of tuberculosis. The largest branches of the renal vein are involved in the tuberculous process. The advanced stage of the lesions in the kidneys justifies the diagnosis of chronic tuberculosis of the kidney, with resulting secondary interstitial nephritis.

Liver.—Countless miliary tubercles occur in sections taken from all parts of the organ. They are for the greater part of smaller size than those of the lung, but show also advanced caseation, reticular formation, and in some instances encapsulation. Giant cells are numerous. There are also many small epithelioid tubercles without caseation. The other changes found are those of moderate chronic passive congestion (nutmeg liver).

Pancreas, stomach, and intestines.—Negative.

Ovaries.—In the left ovary there is present, just beneath the surface, a corpus luteum of oval shape, measuring 1×1 cm. The lutein cells are atrophic and vacuolated. The protoplasm of many is fragmented, and the nucleus does not stain. Within the corpus luteum there are scattered miliary epithelioid nodules showing caseation and numerous giant cells. In one portion several confluent tubercles occur, and these show abundant reticulum and beginning encapsulation. The lutein cells immediately about the tubercles are vacuolated or necrotic. With the exception of two small epithelioid tubercles containing giant cells, but not showing caseation, no tubercles were found in other portions of the left ovary or in any portion of the right. No other pathological changes were found in either ovary.

Tubes.—Negative; no tubercles found.

Uterus.—Uterine wall shows the characteristic changes of the pregnant uterus. No tubercles found in wall. Endometrium gives the appearances of

post-partum decidua at full term. No tubercles found in decidua. In the decidual and subplacental sinuses there are numerous hyaline thrombi characterized by the presence of leucocytes, the nuclei of which are undergoing disintegration. The chromatin of the nuclei is broken into small granules, or in other cases it is represented by diffuse blue staining. The thrombi are composed partly of fibrin and partly of fused red cells (agglutination thrombi). Tubercle bacilli are found in great numbers in these thrombi, and they also lie scattered among the red cells in the placental and decidual sinuses. At the points where the thrombi are adherent to the wall of sinus or vessel slight changes may be found in the wall, either of the nature of a necrosis or beginning epithelioid proliferation, with an occasional characteristic giant cell. No caseating tubercles were found. In some of the larger thrombi the central portion of the hyaline substance shows beginning liquefaction. The majority of the thrombi containing tubercle bacilli, however, present no appearances with ordinary stains that would distinguish them from the hyaline clots and thrombi found in the decidual sinuses under normal conditions, unless it be the almost universal occurrence of a diffuse chromatin-staining in their central portions.

Bladder.—Tuberculous cystitis.

BACTERIOLOGICAL EXAMINATION.

Tubercle bacilli were found in great numbers in the urine taken from the bladder at autopsy, in the caseous material from the kidney, and in the tubercles found in the lung and liver, and in the thrombi of the uterine and decidual sinuses. No bacilli found free in the blood in the vessels in any organ except in the uterus.

PATHOLOGICAL DIAGNOSIS.

Chronic primary tuberculosis of the kidneys; subacute miliary tuberculosis of lungs, liver, and spleen; tuberculous cystitis; tuberculosis of corpus luteum verum; tuberculous thrombosis of decidua; general marasmus; abortion.

FETUS.

(The fetus and placenta were brought to the pathological laboratory as soon as possible after the abortion.)

Male fetus, 25 cm. long, weight about 250 g., attached to middle of placenta by cord 30 cm. long and 5 mm. in diameter. Near the middle of the cord there are several small nodular thickenings. On section these present no pathological changes. The genital organs are perfectly formed. The finger and toe nails are perfect. The head is covered with a fine down; no down on body. The shoulder breadth is 9 cm.; the trochanter distance 4 cm.; circumference of cranium 20 cm. On section the right testis was found in the inguinal canal, the left one just above the internal ring. Careful examination of all the internal organs revealed no macroscopic evidences of tuber-

culosis or other abnormality. The age of the fetus was placed in the late sixth or early seventh month.

About one hundred blood-smears were made from the fetal blood of heart, hepatic vessels, spleen, and umbilical vein. A young and healthy guinea pig was inoculated with a beef-tea suspension of the fetal liver. All of the internal organs were fixed in mercuric chloride, imbedded in paraffin, stained with hematoxylin and eosin for histological study, and with carbol-fuchsin and methylene blue for the presence of tubercle bacilli. Weigert's fibrin stain and other methods were also employed in the study of the thrombi found in the blood-vessels.

MICROSCOPICAL EXAMINATION.

The careful search of sections taken from all the fetal organs failed to reveal any change of structure suggesting tuberculosis. The only pathological changes found were a few small thrombi in the interlobular vessels of the liver. These were hyaline in character, staining deep-red with eosin, and containing but few leucocytes. No necrobiotic changes were observed in the latter. The hyaline material forming the thrombi gave the fibrin reactions only in part, the remaining portion appearing to consist of agglutinated red blood-cells. Staining with carbol-fuchsin showed the presence of tubercle bacilli in the thrombi and also free in the hepatic vessels. No evidences of degeneration or proliferation of the cells of the blood-vessel or tissues in the neighborhood of the thrombi could be found. Staining of the blood-smears made from the fetal liver showed the presence of bacilli in one out of twenty smears. In the blood-smears stained with hematoxylin and eosin the number of megaloblasts and polychromatophile cells seemed greatly increased in numbers as compared with blood-smears taken from another fetus of about the same age.

BACTERIOLOGICAL.

A young and healthy guinea pig was inoculated with a beef-tea suspension of fetal liver on October 22, 1901. The animal showed an increase of temperature and began to lose weight in the second week after the inoculation. The loss of weight and fever continued steadily, and by December 2, the pig appeared very ill; would not move from corner of cage. It was chloroformed and examined immediately after death. Small caseating nodules were found at point of inoculation. In the posterior peritoneal wall there were a number of white nodules of the size of a pin-head. The omentum was rolled up, greatly thickened, and on section showed a cut surface studded with numerous miliary nodules, many of which presented yellowish, softened centers. The mesenteric glands were enlarged, but showed no macroscopic evidences of tuberculosis. The liver, lungs, and spleen were studded with innumerable grayish or yellowish pinhead nodules, many showing a central caseation. Scattered nodules similar to these were also present in the kidneys. The staining of smears made from some of the caseating nodules showed the presence of numerous tubercle bacilli. The microscopical examination of sections of omentum, liver, lungs, spleen, and

kidneys showed the nodules to present the characteristic appearances of miliary tubercles in varying stages of caseation. Staining of the sections with carbol-fuchsin likewise showed the presence of tubercle bacilli in these lesions.

A second pig was inoculated with a beef-tea suspension of the liver of pig No. 1 on December 2. On the next day the animal showed an increase of temperature, and from this time gradually lost weight. On December 18 it gave birth to two young ones. Three days afterward both mother and young died. Autopsy of mother showed a marked generally miliary tuberculosis. This was confirmed by microscopic examination and by the inoculation of a third pig. Other pigs were inoculated with beef-tea suspension of the livers of the two young of pig No. 2. The inoculated animals remained in perfect health months afterwards. No evidences of tuberculosis found in the organs of young.

PATHOLOGICAL DIAGNOSIS.

Bacteriemia (tubercle bacilli) of fetal blood, and agglutination thrombosis without histological changes of tuberculosis.

PLACENTA.

Placenta nearly round, disk-shaped, about 15 cm. in diameter and 3 cm. thick. Cord attached to middle. Naked-eye inspection showed nothing that with certainty could be regarded as pathological. Whitish and yellowish areas, varying in size from a pinhead to that of a cherry, were found on the cut surface, but in every way resembled the small anemic infarcts found in the normal placenta. The organ was cut into parallel strips about 5 mm. in breadth. Half of these were fixed in mercuric chloride and half in 4 per cent. formalin. When after-hardened in alcohol the placental tissue appeared thickly strewn with small grayish spots. The entire organ was imbedded in paraffin. Sections were taken from each block, and many of the latter were wholly cut up into serial sections. Ordinary stains—carbol-fuchsin, Weigert's fibrin stain, polychrome methylene blue, kresyl violet, tri-acid stain, etc., were used in the study of the sections.

MICROSCOPICAL EXAMINATION.

Cord.—Sections showed no histological changes of tuberculosis.

Intervillous spaces.—On examining the sections of the placenta stained with hematoxylin and eosin one is immediately struck by the presence in the intervillous spaces of small, round, hyaline, red-staining masses of a fibrogranular or nearly homogeneous structure, and containing a varying number of leucocytes. They vary in size, but the majority are of about the same dimensions. As seen in the mounted specimen, when held against the light, they are about the size of a small pinhead. In general they resemble the masses of fibrin found in the intervillous spaces of every normal placenta, but at once attract attention by their more regular outline, and by the fact that in nearly every one the leucocytes in the central portion of the mass show a marked karyorrhexis, the disintegration and diffusion of their chromatin

giving a bluish or violet stain to the center of the mass. Closer inspection shows also that many of these small masses contain characteristic giant cells with peripherally arranged nuclei. These giant cells do not resemble the syncytial buds or offshoots; serial sections show that most of them lie in the central part of the hyaline and fibrogranular masses, and are not connected with the villi. In the central portion of the larger masses there is a more finely granular appearance, resembling caseation. The giant cells in some instances are involved in this central disintegration. About the border of the masses the leucocytes stain better, the outlines of their nuclei being preserved.

In the great majority of the smaller masses no cells of epithelioid or fibroblastic type are found. The only elements constituting the mass are: (1) a groundwork of a hyaline, lumpy, or fibrogranular substance staining red with eosin; (2) in the meshes of this, leucocytes showing more or less karyorrhexis; (3) occasional giant cells with peripherally arranged nuclei, in all respects resembling the characteristic giant cells of tuberculous processes. With eosin the ground substance of these masses resembles fibrin; with Weigert's fibrin stain the greater part of it does not stain like fibrin. It stains with various methods as red blood-cells do; and in many cases a direct transition can be seen from red cells forming the periphery of the mass into the hyaline, lumpy substance forming the central portion. The general appearances and the staining reactions of this substance are the same as those of hyaline thrombi composed of red cells; and they correspond in all respects to the agglutination-thrombi described by Flexner.

Staining with carbol-fuchsin and methylene blue shows the presence of varying numbers of tubercle bacilli in all of these thrombi. Free bacilli are also found in the blood of the intervillous spaces.

The agglutination thrombi in some instances lie directly upon the syncytium of the villi, which does not show any perceptible changes. In other cases the syncytial covering of the villus is partly absent, and the thrombus rests directly upon the stroma of the villus. In these cases a proliferation of the cells of the stroma has taken place, and cells of a fibroplastic or epithelioid type have penetrated into the thrombus mass. The appearances are therefore more like those of an epithelioid tubercle. In several instances giant cells of characteristic type are found in the stroma of the villus, apparently arising *in situ*. In many places the syncytium not only does not seem to be affected by the proximity of the tuberculous thrombus, but has evidently extended over and partly surrounded the mass attached to the villus.

Larger areas of thrombosis are found filling up the spaces between a number of villi, the latter showing necrosis. The appearances are similar to those of an ordinary placental infarct, except for the central caseation and the presence of giant cells unlike those of the syncytium. Occasional large areas are found resembling caseous necrosis. Outlines of necrotic villi are seen in the mass, and with Weigert's fibrin stain it is found to be made up of stringy fibrin and of a lumpy, hyaline substance which does not stain like fibrin. With carbol-fuchsin and methylene blue tubercle bacilli in great numbers are found scattered throughout these larger masses. In a few places plume-like colonies of tubercle bacilli are found. Very few giant cells are found in the larger areas.

Chorionic villi.—The villi resemble those of a placenta of about the sixth or seventh month, the stroma showing but little myxomatous character, except in the younger offshoots, that of the larger villi resembling fibrous connective tissue. As mentioned above, tuberculous thrombi are in some cases attached to the syncytium, which shows no apparent changes, or in other cases to the denuded surface of the villus, which shows at such points an epithelioid proliferation with formation of giant cells and beginning caseation. Tubercle bacilli are found in scanty numbers in these, and also rarely free in the intercellular spaces of villi, showing no histological changes. In the chorionic vessels of both large and small size numerous thrombi resembling those of the intervillous spaces are seen. These also contain tubercle bacilli. No changes in the intima of such thrombosed vessels can be made out. In the majority of cases the thrombus is not attached to the vessel wall. On the whole, the villi show relatively slight change, the most striking findings being those of the intervillous spaces. The usual infarcts of the villi are present in perhaps an excessive degree.

Decidua.—The so-called decidual septa, as well as the upper portion of the decidua serotina, show areas of caseation—necrosis, collections of disintegrating leucocytes, and masses of fibrin. In these areas tubercle bacilli are also abundant.

In regard to the origin of the giant cells in the agglutination thrombi, it would seem that the majority are derived from leucocytes. Smaller multinuclear cells resembling transitional cells occur. No evidence could be found of their origin from syncytium. In the villi whose stroma was involved the appearances suggested an origin from the connective-tissue cells.

Bacteriological.—Staining of placental sections showed the presence of great numbers of tubercle bacilli, the majority in the maternal blood, but occurring also in the chorionic tissues and in the fetal circulation.

On October 22 a guinea pig was inoculated intraperitoneally with a thick beef-tea suspension of placenta. At the same time many smears of placental blood were stained for tubercle bacilli, but none were found. The animal became ill within a week, with high temperature and loss of weight, and died on the forty-third day. Unfortunately the body was destroyed by the janitor before an autopsy was made, but the course of the animal's illness leaves practically no doubt as to the nature of its disease.

Pathological diagnosis.—Agglutination thrombi of intervillous spaces due to tubercle bacilli; tuberculosis of chorionic villi; tuberculous thrombi in chorionic vessels.

SUMMARY.

The important features presented by this case are: Woman in fifth month of pregnancy, with chronic tuberculosis of kidney and general miliary tuberculosis; abortion; death; tuberculous thrombosis of placental sinuses and intervillous spaces; tuberculosis of placenta; tuberculous thrombi in fetal blood; presence of free tubercle-bacilli in fetal circulation without histological changes.

A comparison of this case with the other cases of placental tuberculosis reported shows that the changes found in these cases are apparently identical with those described above; viz., that the so-called placental tubercles are for the chief part intervillous thrombi containing giant cells and showing caseation. It would appear that the first steps in the development of placental tuberculosis consists in the formation by tubercle bacilli in the intervillous spaces of thrombi composed of fibrin and agglutinated red cells, and that the villi appear to be secondarily involved. The fact that the thrombi, even when resting upon the syncytium, do not appear to damage it, except in those cases where the villus is almost entirely or wholly surrounded by the thrombus, as well as the more striking fact that the syncytium may even grow out upon or partly around the thrombi, speaks for a marked resisting power on the part of the syncytial layer against the tuberculous process. That this resistance is at last ineffectual is also shown by the destruction of both syncytium and stroma, and by the occurrence of tuberculous proliferation of the cells of the chorionic stroma. It is, therefore, highly probable that the origin of placental tuberculosis is largely or chiefly dependent upon intervillous thrombosis.

The presence of so many tubercle bacilli in the maternal sinuses and intervillous spaces, when none could be found elsewhere in the maternal blood-vessels, is a point of great significance. If tubercle bacilli or other micro-organisms gain access to the maternal circulation, it would seem reasonable to believe that, from the conditions in the placental sinuses—large amount of blood and relatively slow current—they would tend to collect in these blood-spaces and here give rise to pathological disturbances. Such, indeed, seems to have been the case in this instance. Here undoubtedly great numbers of bacilli were free in the general circulation, and these collecting in the placental sinuses, and caught in the fibrin there, multiplied, caused thrombosis, and excited tuberculous proliferation in the chorionic stroma. If such great numbers may collect here in the case of general miliary tuberculosis, is it not also probable that in cases of local or chronic tuberculosis in the mother, in which only a

few bacilli escape into the circulation, these few are also likely to collect in the uterine and placental sinuses? Their further development would depend then wholly upon their ability to multiply under the conditions present there. The germicidal action of the blood may suffice to inhibit or destroy small numbers, and it is probable that only under certain conditions, or when present in the blood in large numbers, their growth is possible.

If multiplication does take place, and agglutination and coagulation of the blood result, it is very probable that both of these processes serve, within certain limits, to protect the placental tissues against tuberculous involvement. If the number of bacilli is very great and many agglutination thrombi are formed, the villi become secondarily involved, possibly in part as the result of circulatory and nutritional disturbances. The microscopical appearances presented by our case certainly convey the impression that the syncytium possesses a greater degree of resistance to tubercle bacilli than does the stroma of the villus. If the latter become infected, it is the result of a passage of bacilli through an intact syncytium or through a surface of the villus denuded of its syncytium. It is impossible to say positively from our studies of this case that the former had actually happened. Villi with apparently intact syncytial covering were found with free tubercle bacilli in the spaces of the stroma; and epithelioid proliferation and giant cells were found under similar conditions. Therefore, it does not seem safe to go so far as to affirm that an intact syncytium will not permit the passage of bacteria.

In any case, this point is not of such great moment if it is true that a denuded chorionic surface will permit the passage of bacteria. It is generally assumed by those who regard the placenta as a secure filter against micro-organisms that the syncytium of the normal placenta is everywhere intact. Such is not the case. From its earliest period the chorion shows progressive atrophy and growth. Anemic infarcts are constantly present, and through such damaged areas bacillary transmission may occur. In the later months of pregnancy the placenta is a senile organ, containing many infarcts and many chorionic villi with damaged syncytium.

There are, therefore, opportunities for the passage of bacteria through such senile villi. In these cases it is very probable that the fibrinous deposit which forms over such villi is a very important factor in preventing such transmission.

As to the fetus, it seems remarkable that so many bacilli could be present in its circulation without causing changes other than those of thrombosis. This may be explained by a more recent passage of the bacilli through the placenta, the time being too short for changes to have occurred; or by the assumption of a diminished virulence on the part of the bacilli; or by a relative or absolute immunity on the part of the fetal tissues. The second consideration may be thrown out, as the inoculation of the guinea pig with fetal blood resulted in an unusually rapid and virulent infection. Further observations and investigations will be necessary to settle the important question of immunity of the fetal tissues. Can it be proved that such an immunity exists, and that tubercle bacilli may live for some time in the fetal tissues without causing histological changes, the theory of latent congenital tuberculosis will receive a tremendous impulse.

It must not be forgotten that evidence favoring this has already been advanced, and that several cases have been reported in which such latency of tuberculosis seemed very probable (see the case above of Doléris and Bourges, 1896). Such a latency in the case of congenital typhoid seems probable in the case described by Blumer, in which a child, born five months after the mother had typhoid, died of typhoid infection nine days after birth. The apparent immunity of the fetal tissues to a number of poisons is also well known.

CONCLUSIONS.

1. In the case of the entrance of tubercle bacilli (miliary tuberculosis or chronic tuberculosis) or other micro-organisms into the maternal blood during pregnancy, the conditions of the circulation in the placental and uterine sinuses favor their collection there.

2. If the bacilli are capable of multiplication, the first step in the development of tuberculosis of the placenta appears to be an

agglutination and coagulation thrombosis of the maternal blood in the intervillous spaces. The formation of such thrombi around the multiplying bacilli may to some degree be protective in inhibiting or restricting their growth.

3. The syncytium appears to possess a certain degree of resistance against tubercle bacilli, the tuberculous thrombi in many cases being attached to an apparently normal syncytium, and occasionally the latter may even be found extending over the thrombus.

4. The question of the passage of tubercle bacilli through an apparently normal syncytium still remains open.

5. In the case of destruction of the syncytial covering, the stroma of the chorionic villi may become involved in a tuberculous process either directly or by an extension from a tuberculous thrombus. The syncytium of the normal placenta is not intact throughout, processes of decay and growth occurring in the chorionic villi from the earliest stages of pregnancy. In the later months the placenta is a *senile* organ, presenting in many places an atrophic or necrotic syncytium (infarcts), and through such damaged areas a passage of micro-organisms may take place. The deposit of fibrin found so frequently over such damaged areas may exert a protective action against such passage.

6. Tubercle bacilli may be found free in the reticular spaces and blood-vessels of the chorionic villi without the occurrence of histological changes of tuberculosis.

7. The fetal blood may contain great numbers of tubercle bacilli without other changes than small agglutination thrombi being present in the fetus. Inoculation shows these bacilli to retain their virulence. From this it may be assumed that the fetal tissues are relatively immune to the action of the tubercle bacillus.

8. Granting such an immunity, it is possible that living tubercle bacilli may be present in the fetus and newborn child without exciting histological changes; and, developing some time after birth, may then give rise to characteristic tuberculous lesions. A true latent congenital tuberculosis therefore is both possible and probable; but additional investigations are necessary

to settle the question of the frequency of such an event. The commonly accepted dicta regarding congenital tuberculosis are probably extreme. It is not at all unlikely that it is of much more common occurrence than is generally supposed.

EXPLANATION OF PLATES VI, VII, VIII.

FIG. 1.—Small agglutination thrombus in intervillous space. Thrombus is composed of red cells, fibrin and leucocytes. Large giant cell near center. Magnification: 135.

FIG. 2.—High-power view of tuberculous thrombus seen in Fig. 1. Magnification: 500.

FIG. 3.—Numerous agglutination thrombi in intervillous spaces. Outlines of red cells can be seen in several. Contained numerous tubercle bacilli. Magnification: 135.

FIG. 4.—Tubercle involving villus. Magnification: 175.

FIG. 5.—Tuberculous thrombus attached to chorionic stem. Syncytium absent at point of attachment, but can be seen extending over and around thrombus. Magnification: 135.

FIG. 6.—Chorionic stem with vessel (at right) containing agglutination thrombus. Numerous tubercle bacilli were found in thrombus. Magnification: 65.

CHANGES IN THE BACTERIAL FLORA OF SEWAGE DURING STORAGE.*

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I. INTRODUCTION.

BACTERIA, like other living things, are within narrow limits adapted to their environment; and in general the different kinds of micro-organisms will be found in fullest activity only under certain rather definite conditions. Each disease has its specific microbe, each different process of decomposition or fermentation is carried on by its own group of bacteria, and water, air, and milk have their more or less constant flora. *A priori*, we might expect to find the same law operating in regard to sewage. To quote from Jordan (1), one of the earliest American investigators of this subject: "The sewage itself—a nutritive medium of varying composition and richness—will contain only those species capable of living and holding their own in the continual struggle for existence. So far as the conditions of life in sewage differ from conditions of life elsewhere, so far will the sewage be inhabited by species peculiarly adapted to those conditions; just as we find, for example, that, among the multitude of bacteria

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taken into the human alimentary canal, only a few species normally find fit conditions for development."

So Laws and Andrewes (4) recognized the fact that sewage, especially when stored, might contain certain characteristic forms. "Sewage may be a favorable medium for the growth of one species, an unfavorable one for another. The latter will, therefore, tend to disappear from older sewage, which will contain larger and larger numbers of those particular organisms for which it is a suitable soil." Such a process as is here postulated has been studied in the case of milk by H. W. Conn and W. M. Esten (15), with very striking results. They find that, on allowing milk to stand at the room temperature, there is an enormous increase in the total number of bacteria present, which at first are of many different kinds. Gradually, however, the percentage of lactic acid forming bacteria rises, until often it reaches as high as 99 per cent. Thus in the struggle for existence those best fitted to survive and multiply have overrun the rest, and there results an almost pure culture of a single group.

Experimental evidence as to the existence of true sewage bacteria in any such sense as this has, heretofore, been meager. Numerous observers have recorded the total number of bacteria present in crude sewage; but without reference to their kinds. Laws and Andrewes (4), for example, report from two to eleven million per cubic centimeter in various London sewages, while Clowes (9) found three million and a half at the Crossness outfall, and nearly four million at Barking. The number of bacteria in American sewage is somewhat less. The average of the bacteriological analyses of the Lawrence Street sewage from 1894 to 1901, as published in the annual reports of the Massachusetts State Board of Health, was 2,800,000; and two twenty-four hour examinations of Boston sewage, made at the Sanitary Research Laboratory of the Massachusetts Institute of Technology during the summer of 1903, gave an average of 3,660,000. It is also well recognized that when sewage is stored under favorable conditions these numbers are greatly increased. Laws and Andrewes (4) found 33,800,000 bacteria per cubic centimeter after three days' storage of London sewage in a glass bottle and an interest-

ing series of chemical and bacteriological analyses of stored sewage, made by the Massachusetts State Board of Health in 1894(3), showed the relations still more clearly. The bacteriological results of the experiment were as follows:

TABLE I.

Number of Bacteria per Cubic Centimeter in Fresh and Stored Sewage
(*XXVI. Ann. Rep. Mass. S. B. H. for 1894*).

Day	Hour	Bacteria	Day	Hour	Bacteria
Mar. 11	10:30 A. M.	1,190,000	Mar. 13	10:30 A. M.	12,810,000
11	12:30 P. M.	1,085,000	14	10:30 A. M.	11,235,000
11	3:00 P. M.	1,505,000	15	10:30 A. M.	6,825,000
11	6:00 P. M.	1,530,000	16	10:30 A. M.	4,485,000
12	8:00 A. M.	20,475,000	18	10:30 A. M.	3,420,000
12	12:00 M.	23,100,000	19	10:30 A. M.	2,341,000
12	5:00 P. M.	20,000,000			

This examination shows a marked increase in the total number of bacteria up to twenty-five and one-half hours, at which time there were twenty times as many bacteria present as in the fresh sewage, followed by a gradual decrease in numbers to the end of the week.

The figures quoted throw no light, on the nature of the organisms present; and, on the other hand, those observers who have isolated and described certain species of bacteria found in sewage have usually ignored the relative preponderance of the different types. Jordan (1) at Lawrence, Roscoe and Lunt (2), Laws and Andrewes (4), and Clowes and Houston (10) in England have recorded some fifty species from this source. The last two papers cited, as well as the first report of Clowes (9) to the London County Council, contain, some figures which are both qualitative and quantitative as regards certain intestinal bacteria, liquefiers, anaërobies, etc.; and Klein and Houston in the reports of the Local Government Board (8), (11), (12) and of the Royal Sewage Commission (22), (23) give numerous determinations of the numbers of colon bacilli, streptococci, and spore-bearing anaërobies. These investigators have, however, only studied certain special organisms in fresh sewage; so far as we are aware, no attempt has

been made to gain a general idea of the relation of all the different groups of bacteria in that and of the change in relation during storage.

Information of this nature would seem to have a practical as well as a theoretical value in view of the complex character of the processes of sewage disposal as they have been developed during recent years. The so-called "bacterial methods" of sewage purification are attempts to intensify the process which goes on in an ordinary sand filter, by dividing it into stages, in each of which a special group of bacteria may find conditions especially favorable to its activity. Thus Rideal (18) distinguishes three distinct stages—the anaërobic, the semi-anaërobic, and the aërobic—carried out respectively in the septic tank and the first and second contact filters. Obviously, under such conditions, an exact knowledge of the organisms concerned is highly desirable; but although we know some of the characteristics of the nitrifying organisms associated with the two later stages of purification, of the anaërobic forms which work in the septic tank we are almost wholly ignorant.

It seemed to the authors that a quantitative study of the groups of bacteria in fresh and stored sewage, especially if any tendency to the formation of a pure culture should appear, might throw important light on the forms active in sewage purification. Furthermore, the fate of the more typical intestinal bacteria in stored sewage is of interest to the student of the etiology of the infectious diseases. This examination of the bacterial flora of sewage was, therefore, undertaken with two ends in view—to determine whether any groups of bacteria multiply in stored sewage in such fashion as to be considered characteristic of it, and whether parasitic organisms find that medium favorable or inimical to their development.

II. METHODS.

1. *Technic of isolation.*—The samples of sewage studied in this investigation, six in number, were collected at different times during the winter of 1902–3 from one of the smaller Boston sewers through a manhole on the Dartmouth Street side of Cop-

ley Square. The contributing area of some eight acres includes three large hotels, besides residences and apartment houses, and has a population of probably a thousand persons. A very fresh domestic sewage was thus obtained.

The samples were collected by lowering a sterile pail into the sewer and filling from this a sterile gallon bottle about three-fourths full. The latter was immediately taken to the laboratory and the analysis of the fresh sewage begun within one-half hour of the time of collection. The sewage was then stored in the gallon glass bottle with a tight glass stopper, in the dark, at room temperature, and analyses were made at different periods of storage—as twelve, twenty-four, forty-eight hours, seven days, and a month.

Conn and Esten (15), in the work quoted above, based their determination of the groups of bacteria merely on the cultural differences exhibited by colonies on the litmus-lactose-gelatin plate. Our attempt has been to carry out a more exhaustive study by plating the sewage in great dilution (about twenty colonies appearing on the plate), so that all the colonies could be isolated and worked out in detail. To assume that such a small number of bacteria selected at random will be strictly representative of the great multitude present in the sewage is, of course, erroneous. The greatest care was exercised, however, to secure a thorough mixture before sampling; and the concordant results obtained appear to justify the general accuracy of the method.

The agar streak cultures obtained from the original plate were at once examined in sub-cultures without the preliminary cultivation, recommended by so many observers—as by Fuller and Johnson (14), and Jordan (24), in their work on the classification of water bacteria. In many cases this is undoubtedly a valuable help, since thus differences in past environment may be eliminated; but in our work it was desired to isolate the different forms in the condition in which they occurred in the sewage as nearly as possible.

In each experiment samples of sewage were plated in parallel under aerobic and anaerobic conditions. For the former purpose standard nutrient gelatin was used, made up according to the recommendation of the Committee on Standard Methods of Water

Analysis of the American Public Health Association (19), and the plates were kept at room temperature as long as possible without having the liquefiers overrun other colonies and thus prevent the isolation of pure cultures. We planned at first to make a series of parallel isolations using lactose agar at 37° , since in an earlier investigation very high numbers of streptococci had been found by this process in the effluent of a septic tank. Preliminary experiments, however, did not indicate any marked difference in the sorts of bacteria developing from raw sewage on these two media. For Samples I, III, and IV, therefore, only gelatin plates were made. For Samples II and V total counts were also obtained on lactose agar at 37° , but the gelatin plates were used for qualitative studies. In the case of Sample VI, plates were made on gelatin, and lactose agar at 37° and on agar at 37° , for total counts; and the lactose agar plates were used for the isolation of the subcultures to be examined.

For cultivation under anaërobic conditions, neutral agar was used instead of the ordinary agar made up with an acidity of 1 per cent., since Dr. J. H. Wright has pointed out that this is more favorable for the development of strict anaërobes. After some experimentation with Buchner's tube and with the glass-plate method of Sanfelice, as described by Hunziker (21), the method suggested by Wright (16) was found to be most satisfactory. After pushing a plug of sterile cotton one-third way down an ordinary culture tube, dry pyrogalllic acid was poured in, a few centimeters of strong caustic hydrate added, and the whole sealed with a rubber stopper. Instead, however, of rolling the tubes after the fashion of Esmarch, it was found simpler and more satisfactory to lay them in a horizontal position and allow the agar to set in a flat layer a quarter of an inch thick. After incubation this slab of agar could be slid out of the tube entire and streaks made from the colonies with ease. Two cultures were made from each colony, one under aërobic, one under anaërobic conditions. If development occurred on the former streak, the organism was not further studied, since facultative forms were presumably represented on the gelatin plate, and this process was intended only to detect obligate anaërobes.

2. *System of classification.*—The elaborate study of minute specific differences was obviously impossible in a work of this character, and of doubtful advantage even if feasible. There is much truth in the suggestion of Marshall Ward (7) that “the ‘species’ of the descriptive handbooks—principally medical—are frequently not species at all, in the botanical sense, but varieties, or growth-forms, the distinctive characters of which are not constant. These so-called species need revision and grouping around types, which may turn out to be the true species.” We have, therefore, only attempted to place the organisms isolated in certain large and well-marked groups. Flügge (6) laid the foundation for a natural classification of the bacteria; Ward (7), Fuller and Johnson (14), Weston and Kendall (20), and Jordan (24) for water bacteria, and Conn (13) for milk bacteria, have developed more detailed groupings. In our work we adopted the system of Fuller and Johnson, with certain modifications. The main difference consists in the recognition of morphological characters; we have included groups for the cocci and spirilla, have characterized the *B. subtilis* group by the presence of spores on potato, and have separated the non-fluorescent, non-chromogenic, non-liquefying bacteria into the *B. coli* and *B. aërogenes* series according to motility. The system as thus modified is as follows:

DEFINITION OF GROUPS.

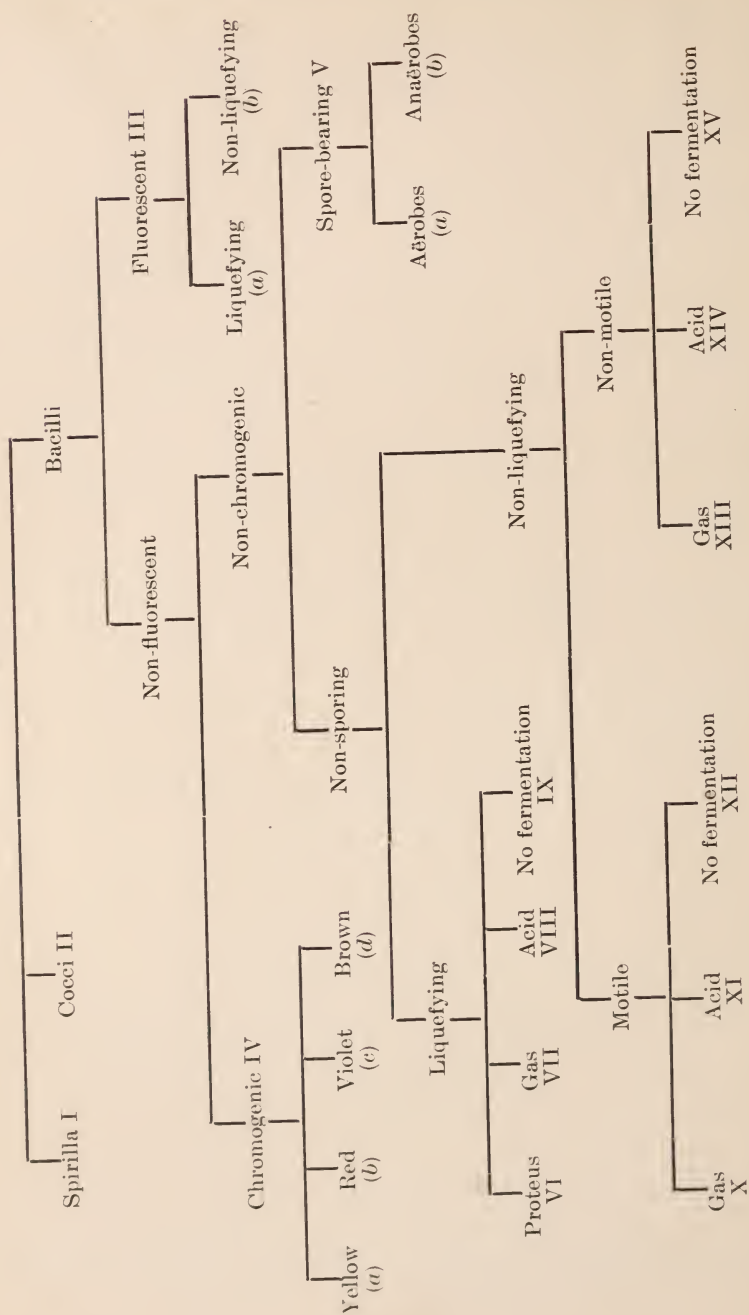
- Group I. *Spirillum undula* type. All spirilla.
- Group II. *Streptococcus pyogenes* type. All cocci.
- Group III. *B. fluorescens* type.
 - a) Fluorescent forms, liquefying gelatin.
 - b) Fluorescent forms, not liquefying gelatin.
- Group IV. Chromogenic forms.
 - a) Yellow and orange; *B. subflavus* type.
 - b) Red; *B. prodigiosus* type.
 - c) Violet; *B. janthinus* type.
 - d) Brown; *B. brunneus* type.
- Group V. Spore-bearing bacilli.
 - a) Aërobes; *B. subtilis* type.
 - b) Anaërobes; *B. sporogenes* type.
- Group VI. *B. vulgaris* type— all non-spore-forming, non-fluorescent, non-chromogenic gelatin-liquefying bacteria forming proteus-like colonies on gelatin.

- Group VII. *B. cloacae* type—all non-spore-forming, non-fluorescent, non-chromogenic bacilli, not forming proteus-like colonies liquefying gelatin and fermenting dextrose with the formation of gas.
- Group VIII. *B. liquidus* type—same as Group VII, except that dextrose is fermented without the formation of gas.
- Group IX. *B. superficialis* type—same, except that no fermentation of dextrose occurs.
- Group X. *B. coli* type—all non-spore-forming, non-fluorescent, non-chromogenic, non-liquefying bacilli, which are motile and ferment dextrose with the formation of gas.
- Group XI. *B. typhi* type—same as Group X, except that fermentation of dextrose occurs without formation of gas.
- Group XII. *B. candicans* type—same, except that no fermentation of dextrose occurs.
- Group XIII. *B. aërogenes* type—all non-spore-forming, non-fluorescent, non-chromogenic, non-liquefying, non-motile bacilli, which ferment dextrose with the formation of gas.
- Group XIV. *B. ubiquitus* type—same as Group XIII, except that fermentation of dextrose occurs without the formation of gas.
- Group XV. *B. rhinoscleromatis* type—same, except that dextrose is not fermented.

The relation of the various groups is better shown in diagrammatic form on following page.

3. *Methods of studying cultures.*—The classification outlined above necessitated the use of four different media—agar, gelatin, potato, and dextrose broth—all prepared according to the standard procedure of the Committee on Water Analysis of the A. P. H. A. The cultures, first isolated on agar streaks, were kept at 20° as stock cultures for the inoculation of other media, and upon them the general morphology of the organism and its fluorescence were observed. Next a potato culture was made for chromogenesis, and this was examined after a week's incubation at 20° for spores. Thirdly, a gelatin stab furnished information as to the characteristic form of surface growth and the presence or absence of liquefaction, the tubes being kept ten days for the observation of the latter character. Finally a dextrose tube incubated for three days at 37° (all the bacteria tested for gas production grew well at this temperature) revealed the fermentative powers of the organism.

TABLE II.



The separation of the *B. coli* and *B. aërogenes* series proved the most troublesome feature of the examination. According to Flügge, the distinction lies in the fact that organisms of the former type are motile and produce an expanded disk-like surface growth on gelatin, while *B. aërogenes* is non-motile and forms small drop-like colonies. We found, however, that no constant relation existed between these characteristics, and thereafter laid stress only on the motility, as determined by examination of an agar streak cultivated for twenty-four hours at 37°. In most of our experiments the cultures were subjected to preliminary cultivation in three successive broth tubes before this determination. It would be an obvious convenience if in some medium the motility of an organism could be determined without resorting to the microscope; and we hoped that this might be accomplished by the use of a low-percentage gelatin having a lower viscosity than ordinary gelatin. In a gelatin stab the motile forms might be expected to spread through the medium, while the others would be confined to a growth in the region of the needle. It was found that 2 per cent. gelatin just remained solidified at the room temperature, and this was used for experiment. When the stabs were kept in the ordinary position, no difference was noticed between the motile and the non-motile forms. When, however, the tubes were turned upside down, it was found that the motile organisms tended to spread diffusely upward through the gelatin, while the non-motile forms gave ordinary needle growths. Experiments with this medium were begun only toward the close of our work, and they were not carried far enough to warrant definite conclusions.

III. RESULTS.

1. *Total number on various media.*—The general results of the counts made upon the various media are shown in Table III.

In each case, except Sample III, where the maximum was reached in twelve hours with a slight falling off in twenty-four hours, there was an increase in total numbers up to twenty-four hours, and then a steady decrease up to at least six months. The number of facultative anaërobes was determined from the anaërobic agar tubes, those colonies isolated from this source and developing

on the aërobic agar streak being classed under this head. These behaved in much the same way as did the bacteria on the gelatin plate, except that they reached a maximum at about forty-eight instead of twenty-four hours and did not fall off so rapidly as the aërobes.

TABLE III.

Number of Bacteria per Cubic Centimeter in Fresh and Stored Sewage.

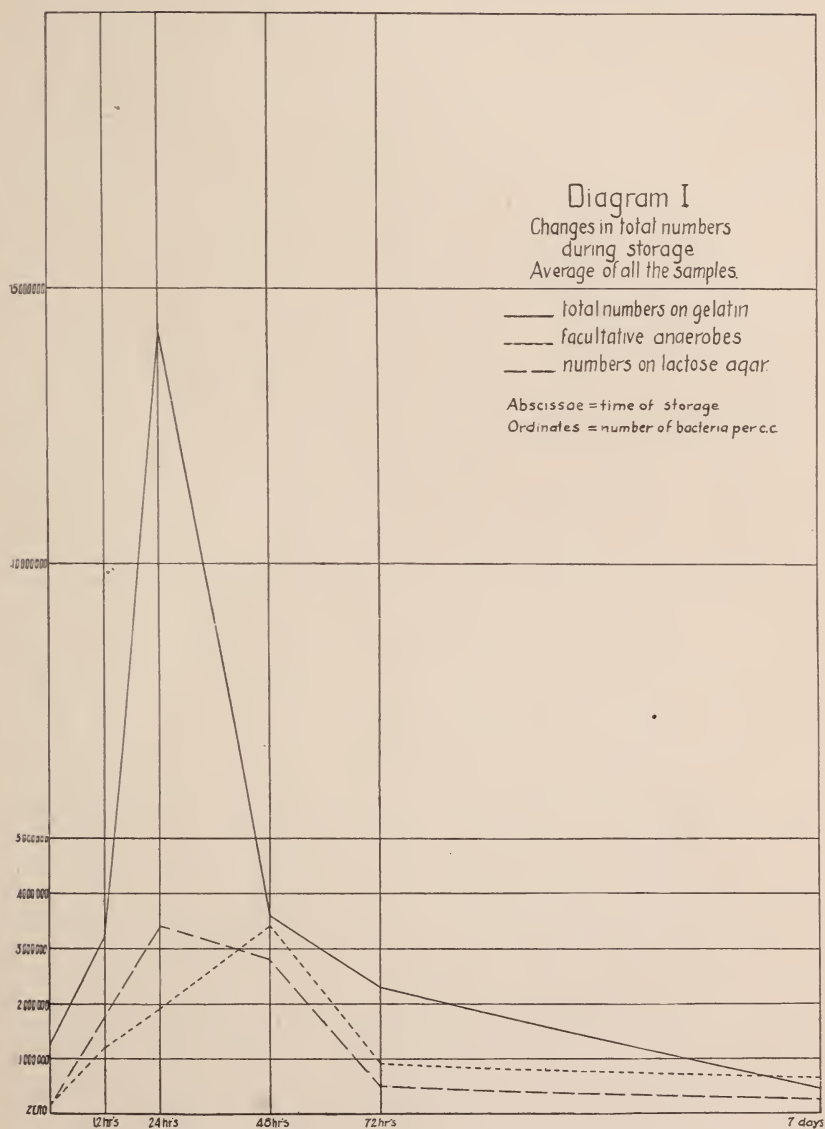
Sample	Storage	Gelatin at 20°	Lactose Agar at 37°	Agar at 37°	Anaërobic Agar
I.....	Fresh	300,000
II.....	Fresh	1,200,000	125,000
III.....	Fresh	500,000	130,000
	12 hours	4,200,000	720,000
	24 hours	3,000,000	1,200,000
	7 days	650,000	500,000
	28 days	64,000	10,000
	5½ months	23,000	1,700	240
IV.....	Fresh	800,000	140,000
	12 hours	2,200,000	1,700,000
	24 hours	13,500,000	1,900,000
	30 days	80,000	32,000
V.....	Fresh	3,100,000	150,000	150,000
	24 hours	23,000,000	2,800,000	2,600,000
	48 hours	3,600,000	2,815,000	3,400,000
	72 hours	2,300,000	500,000	900,000
	7 days	360,000	800,000
VI.....	Fresh	1,550,000	180,000	500,000
	24 hours	17,600,000	4,000,000	16,700,000
	7 days	695,000	220,000	400,000

The figures for the different periods, obtained by averaging all the samples are given in Table IV, and the relations are shown graphically in Diagram I.

TABLE IV.

Number of Bacteria per Cubic Centimeter in Fresh and Stored Sewage (Average of All Samples).

Storage	Gelatin at 20°	Lactose Agar at 37°	Agar at 37°	Anaërobic Agar
Fresh.....	1,240,000	151,000	500,000	140,000
Twelve hours.....	3,200,000	1,210,000
Twenty-four hours.....	14,200,000	3,400,000	16,700,000	1,900,000
Forty-eight hours.....	3,600,000	2,815,000	3,400,000
Seventy-two hours.....	2,300,000	500,000	900,000
Seven days.....	440,000	220,000	400,000	650,000
Twenty-eight to thirty days.....	72,000	21,000
Five and one-half months	23,000	1,700	240



The sharp rise, with a subsequent more gradual decline, is well marked in all the curves of Diagram I; and, as in the Lawrence experiment cited above, the maximum number of bacteria was reached after about twenty-four hours. Again, it is evident by a comparison of the curves that during the first twenty-four hours the multiplication was mainly confined to the strictly aërobic bacteria, since the number of anaërobes remained comparatively insignificant. The latter, however, continued to multiply after the total number had begun to fall off and reached their maximum in forty-eight hours; at that and all subsequent periods the proportion of anaërobes was a considerable one. This corresponds with the common idea that the first fermentation of sewage is aërobic, but that the facultative anaërobes become more and more important as the oxygen is consumed.

2. *Numbers in the separate groups.*—The bacteria isolated from the various samples of sewage are classified by groups in Table V. With the exception of the violet chromogenes, the strict anaërobes, and the proteus organisms, all our groups were represented, and no one form appeared specially predominant either in the raw or septic sewage. We had expected that some one or two types might show a disproportionate increase as the sewage aged, but this appeared not to be the case. Table VI, where the number of bacteria in each group is expressed in its percentage of the total number, makes this still more clear. With the exception of a preponderance of the *B. coli* group in Sample VI, the various types of bacteria occurred in fairly equal proportions, the occasional deviation being within the limits of error due to the difficulty of securing an average sample.

Table VII has been compiled by averaging the figures obtained for the more important groups in the analysis of the first five samples, Sample VI having been omitted since the use of lactose agar at 37° as the medium for isolation made the latter not strictly comparable. Diagram II shows the relation of the different types of bacteria during the first week of storage in graphic form. The individual groups in almost every case show the same tendency as the total numbers; that is, a steady increase up to twenty-four hours, and then a gradual falling off until, at the end of the week, the

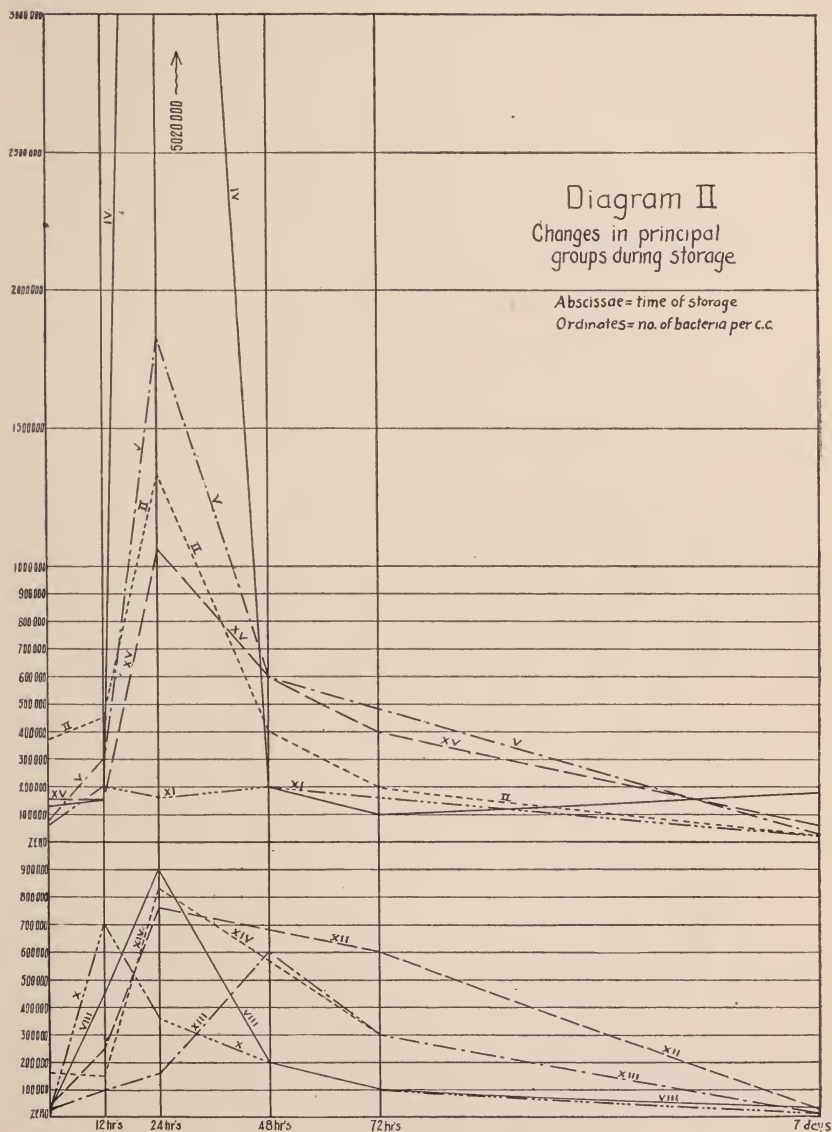


TABLE V.
Bacteria per Cubic Centimeter in Each Group.
(Last three ciphers omitted.)

DATE OF COLLECTION	SAMPLE	STORAGE	DILUTION	TOTAL NUMBER	I	II	BACILLI													YEASTS, MOLDS, ETC.										
							FLUO- RESCENT	CHROMOGENIC				NON-FLUORESCENT																		
								LIQUEFYING	NON-LIQUEFYING	SPORE- BEAR'G	Yellow	Red	Violet	Brown	Aërobic	Anaërobic	LIQUEFYING GELATIN				No SPORES			NON-CHROMOGENIC						
																	Proteus	Gas	Acid		No Ferm.	Gas	Acid	No Ferm.	Gas	Acid	No Ferm.	Gas	Acid	No Ferm.
Oct. 8 24	I	Cocci	50 700	300 1200	50 1200	50 700	20 50	30	80 100	40 50	30	10	20 50				
Nov. 19	III	Fresh	160	500	400	160	20	20	60	1000	400	...	20	120	...	60				
		12 hours	400	2000	400	600	600	200	200	200				
		24 hours	400	3000	400	800	800				
		28 days	4	650	50	50	50			
Dec. 10	IV	5½ months	1	223				
		Fresh	150	800	500	150	50	50				
		12 hours	500	2200	500	400	400	100	100	100			
		30 days	1000	13500	1000	500	500	500	500	500			
Mar. 4	V	Fresh	800	3100	800	50	100				
		24 hours	2000	23000	2000	200	200	1000	2000	800			
		48 hours	400	3600	400	200	200	600	600	600			
		72 hours	200	2300	200	100	100	300	300	400			
Apr. 9	VI	7 days	40	360	40	20	20				
		Fresh	180	180	180	80	10	30			
		24 hours	200	4000	200	2200	800	800	200	10			
		7 days	10	220	10	110	10	10			

TABLE VI.
Per Cent. of Bacteria in Each Group to Total Number.

DATE OF COLLECTION	SAMPLE	STORAGE	TOTAL NUMBER	I	II	BACILLI										YEASTS, MOLDS, ETC.							
						FLUO-RESCENT		CHROMOGENIC			NON-CHROMOGENIC						NON-FLUORESCENT						
						LIQUEFYING	Non-LIQUEFYING	Yellow	Red	Violet	Brown	SPORE-BEARING	LIQUEFYING GELATIN				MOTILE			NON-LIQUEFYING			
													Proteins	Gas	Acid		No Ferm.	Gas	Acid	No Ferm.	Gas	Acid	No Ferm.
Oct. 8, 24.....	I	Fresh	100	16.5	100	10.0	25.0	13.0	4.0	10.0	3.0	6.5	4.0				
Nov. 19.....	II	Fresh	100	57.5	8.0	4.0	4.0				
	III	Fresh	100	32.0	4.0	12.0	4.0	24.0	12.0				
	IV	12 hours	100	9.5	9.5	4.5	14.0	24.0	9.5	9.5	4.5	4.5	6.5				
	V	24 hours	100	13.0	100	13.0	7.5	6.5	20.0				
	VI	7 days	100	25.0	7.5	7.5	6.0	7.5				
Dec. 10....	VI	28 days	100	18.0	6.0	6.0	25.0				
	VI	5 1/2 months	100	4.5	9.0	4.5	9.0	9.0	4.5	13.0	9.0	9.0	21.5	6.0				
	IV	Fresh	100	18.5	12.5	18.5	6.0	6.0	6.0	6.0	6.0	6.0	6.0				
	IV	12 hours	100	22.5	4.5	9.0	4.5	13.5	4.5	18.0	3.5	3.5	4.5	4.5	4.5	4.5	4.5				
	IV	24 hours	100	7.5	3.5	12.0	12.0	11.0	3.5	6.0	6.0	6.0	12.0	12.0	12.0				
Mar. 4	V	30 days	100	6.0	6.0				
	V	Fresh	100	26.0	6.5	20.0	3.0	6.5	3.0	3.0	25.0	18.0				
	V	24 hours	100	8.5	43.0	8.5	8.5				
	V	48 hours	100	5.5	5.5	16.5	5.5	5.5	5.5	5.5	16.5	16.5	16.5	16.5				
	V	72 hours	100	8.5	4.5	4.5	4.5	4.5	4.5	4.5	25.0	12.5	12.5	17.0	4.5				
Apr. 9.....	V	7 days	100	11.0	5.5	5.5	5.5	11.0	3.5	3.5	5.5	5.5				
	VI	Fresh	100	22.0	45.0	5.5	5.5	16.5	16.5				
		24 hours	100	100	4.5	55.0	20.0	4.5	26.0	9.0	4.5				
		7 days	100	50.0	4.5				

numbers are smaller than in the fresh sewage. Thus it is seen that the changes in the different groups are very nearly parallel and there is a close harmony between the various samples, the different groups occurring with about the same frequency in each. The chromogenic forms show the most marked development followed by the cocci and the spore-bearing aërobes. Group X reaches a maximum before the rest, Group XIII after; Group XI alone does not multiply appreciably. But, as far as our results go (and they are singularly concordant, when the possible sources of error are considered), there appears to be no tendency toward the predominance of any special form in stored sewage.

TABLE VII.

Number of Bacteria per Cubic Centimeter in Principal Groups (Average of All Samples).

(Last three ciphers omitted.)

Storage	II	IV	V (a)	VIII	X	XI	XII	XIII	XIV	XV
Fresh.....	372	128	74.5	30	28	60	44	30	162	154
12 hours....	450	150	300	450	700	200	250	100	150	150
24 hours....	1,330	5,020	1,830	900	360	160	760	160	830	1,060
48 hours....	400	200	600	200	200	200	600	600
72 hours....	200	100	100	100	600	300	300	400
7 days.....	20	180	25	35	10	20	25	10	60
1 month.....	2.5	10.5	2	7	4.5	10.5	5	5
5½ months..	1	3	2	1	3	2	5

3. *Special intestinal forms.*

a) *Anaërobes*.—We were at first surprised at the entire absence of obligate anaërobes from the tubes used for routine analysis, as described above, especially in view of the importance assigned to the *B. sporogenes* by the English sanitarians. Klein and Houston (8), (12) record from thirty to five thousand of these organisms per cubic centimeter in crude sewage. Houston (23) states that their number is usually 1,000 and not uncommonly 10,000 per cubic centimeter; and it might naturally be expected that on storage obligate anaërobes would multiply still further. We therefore examined measured portions of Samples III and IV by Klein's milk method. This consists, it will be remembered, in inoculating sterile milk with the material to be tested, heating to 80° for ten to fifteen minutes, to destroy vegetative cells, and

incubating under anaërobic conditions for thirty-six hours at 37°. If *B. sporogenes* or similar spore-bearing anaërobes be present, the milk will be found changed in a characteristic manner. "The cream is torn or altogether dissociated by the development of gas, so that the surface of the medium is covered with stringy, pinkish-white masses of coagulated casein, inclosing a number of gas bubbles. The main portion of the tube formerly occupied by the milk now contains a colorless, thin, watery whey, with a few casein lumps adhering here and there to the sides of the tube. When the tube is opened the whey has a smell of butyric acid, and is acid in reaction. Under the microscope the whey is found to contain numerous rods, some motile, others motionless" (17).

The results of our examination were as follows:

SAMPLE IV.

Fresh.—Typical milk change given in dilutions 1:10 and 1:100, but not in 1:1,000; showing at least 100, but less than 1,000 spores per cubic centimeter.

Twelve hours' storage.—Typical change in dilutions 1:50, 1:100, and 1:1,000, but not in 1:10,000; showing at least 1,000 spores per cubic centimeter.

Twenty-four hours' storage.—Typical change in 1:50, 1:100, and 1:1,000, but not in 1:10,000; showing at least 1,000 spores per cubic centimeter.

Thirty days' storage.—Typical change not shown in 1:10 or 1:100; showing less than 10 spores per cubic centimeter.

SAMPLE III.

Five and one-half months' storage.—Typical change in both of two tubes inoculated with 1 c.c. each, in one out of three diluted 1:10, in one out of three diluted 1:100, but no change in three tubes at 1:1,000 dilution; showing between 10 and 100 spores per cubic centimeter.

It is obvious why the *B. sporogenes* group was not represented in our routine studies, since the facultative forms were so abundant that a dilution of 1:10,000 had to be used for isolation, and even as measured by the more sensitive milk test, the *B. sporogenes* group was never sufficiently abundant to be detected in such dilution. Furthermore, of the organisms giving Klein's typical change in milk, not all are obligate anaërobes. Some at least of those found in our investigation were facultative forms.

b) *B. coli* group.—The organisms of the colon group are of special interest, both from their adoption by the water analyst as a measure of pollution, and from the probability that their behavior in sewage may throw some light on that of the typhoid bacillus under similar conditions. Klein and Houston (8) (12) record from 90,000 to 2,000,000 of these organisms in fresh sewage, and Houston (23) gives 100,000 as a normal number. Klein (5) states that “*B. coli* retains for a long time in sewage its vitality and its power of self-multiplication.”

In most of our experiments this group of organisms was not found in such large numbers as we had expected. In two samples of fresh sewage they were absent (dilutions of 1 : 20,000 and 1 : 100,000), and in the other cases we found 40,000, 50,000, and 60,000 per cubic centimeter, respectively. Like the other bacteria, this group showed a rapid multiplication in the first twenty-four hours, followed by a decline rather more marked than was exhibited by most of the other groups. The maximum for the colon bacilli appeared to be reached earlier than in other cases, and in both Samples III and IV these organisms made up about a fifth of the total after twelve hours' storage. In Samples III, IV, and V, however, the *B. coli* group was only once represented at the end of a week, and not at all after a month.

Sample VI stands out in marked contrast to all the others, since the colon group made up half of the total at all the three periods of analysis, 110,000 per cubic centimeter being found even in the week-old sample. The use of lactose agar at 37° instead of gelatin at 20° as the medium for the original isolation of the organisms studied in this case may have affected this result; or it may simply represent a chance variation in the flora of the sample as collected.

c) *Streptococci*.—The importance of the streptococci as sewage organisms has been fairly well established in recent years since Laws and Andrewes (4) in 1894 found a small streptococcus to be the most abundant organism in several samples of hospital sewage. Houston (11) (22) records the number of streptococci in crude sewage as at least 1,000 per cubic centimeter, and in the

last paper cited states that "they are delicate micro-organisms, and readily lose their vitality and die. They probably are little prone to enter on a saprophytic phase or to multiply to any great extent, if at all, under such conditions."

On the contrary, Horrocks (17) holds an opposite opinion, and declares that "the sewage streptococci appear to maintain their vitality in sewage for a much longer time than *B. coli*."

Our Group II, though including all spherical bacteria, was mainly made up of the sewage streptococci, only a few chromogenic staphylococci being found. As will be seen by reference to Table VI, it accounted for 18-57 per cent. of the bacterial content of the fresh sewage, and increased rapidly in the first twenty-four hours, though not retaining its original relative predominance. Cocci were found in appreciable numbers after thirty days in Sample IV and after five months in Sample III, apparently supporting the view of Horrocks rather than that of Houston.

d) *Other special groups*.—Most of the observers who have studied sewage bacteria record *Proteus* forms as abundant. Clowes and Houston (10), for example found in several cases at least 100,000 per cubic centimeter in fresh sewage. No organisms producing gas, liquefying gelatin, and forming the characteristic surface growth were, however, found in our investigation. In fact, there was a surprising scarcity of liquefying forms of any kind. The *B. liquidus* type (Group VIII) was indeed, one of the most constant forms in the stored sewage, but never rose to high numbers. *B. fluorescens*, sometimes—probably erroneously—described as a sewage form, was found only once in fresh and a very few times in the stored sewage.

On the other hand, the brown chromogenes (Group IVd), the *B. subtilis* type (Group Va) the *B. typhi* type (Group XI), the *B. candicans* type (Group XII), and the *B. rhinoscleromatis* type (Group XV) were constant inhabitants of the sewage, all but Group XI showing the typical change during storage which is common to all the groups.

IV. CONCLUSIONS.

1. The bacteria in Boston sewage include a wide diversity of forms, the most noticeable groups represented being the cocci, the chromogenes, the *B. subtilis* group, the *B. coli* group, the *B. aërogenes* group, and the *B. rhinoscleromatis* group.

2. The total number of bacteria in stored sewage rises rapidly at first, increasing more than tenfold in twenty-four hours, and then decreasing for at least six months.

3. The maximum of the obligate aërobes is reached after twenty-four hours, after which they decrease rapidly, while the facultative anaërobes continue to increase up to forty-eight hours, and after that period maintain a predominance. Obligate anaërobes are at no time abundant.

4. The rise and fall of the bacteria in stored sewage appear to affect the various groups of organisms in an almost equal degree. All first increase and then decrease in number, the multiplication of the chromogene, the cocci, and the *B. subtilis* group being most marked.

5. Sewage does not appear to be a more unfavorable medium for the intestinal bacteria than for other forms. Organisms of the *B. coli* type, at first present to the number of some 50,000 per cubic centimeter, multiply with the others, and afterward may persist in considerable numbers up to seven days.

6. If this work is confirmed it would seem that there is no tendency toward the development of a pure culture of any dominant forms in stored sewage; and it seems questionable whether the term "sewage bacteria" signifies anything more than a chance mixture of organisms derived from a wide variety of sources.

In conclusion the authors desire to express their gratitude to Professor W. T. Sedgwick for his interest and advice in the prosecution of this work.

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ON SOME CULTURAL RELATIONS AND ANTAGONISMS
OF *BACILLUS COLI* AND HOUSTON'S SEWAGE
STREPTOCOCCI; WITH A METHOD FOR THE
DETECTION AND SEPARATION OF THESE MICRO-
ORGANISMS IN POLLUTED WATERS.*

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IT is generally believed that the occurrence of *B. coli* in considerable numbers in water supplies is an indication of fecal pollution and renders the water unsuitable for drinking purposes. The opinion that this organism is characteristic of pollution from human sources was long since proved to be erroneous, as it was shown by Dyar and Keith (1), Smith (2), Flint (2A), Belitzer (3), and Moore and Wright (4), to be constantly present in the intestines of many groups of animals. More recently a number of investigators in Europe and America have reported that bacilli behaving in all respects like the colon bacillus of the human intestine are frequently found in nature where there is no evidence of direct or immediate fecal pollution. Kruse (5) and Weissenfeld (6) declared that *B. coli* is present in almost all waters, good or bad. One of us (7, 8) has isolated from grains and products of milling a considerable number of organisms having all the characteristics of *B. coli*, and Papasotiriu (9) obtained the same results in Europe in an investigation of similar scope. Other workers have also published results which lead to the conclusion that bacteria corresponding in every way to *B. coli* are by no means confined even to animal intestines, but are widely distributed in nature. While this fact has not yet destroyed altogether the value of the "colon test" for potable waters, it has made it clear that extreme care should be used in interpreting the results thereby obtained, as has already been pointed out by Freudenreich (10), Smith (11), and one of us (12); and it has also led to a belief in some quarters that, if possible,

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confirmatory evidence of some sort should be adduced whereby the bacteriologist may establish beyond doubt recent intestinal pollution.

Such a means of confirmation seems to be offered by the streptococci of Houston, which have been shown by various workers to be present in sewage, sewage effluents, and polluted waters, and in soil which has received the wastes of animal life, but to be absent from unpolluted waters and virgin soils. Originally reported by Laws and Andrewes (12) in 1894, the importance of these micro-organisms was not emphasized until 1899 and 1900, when Houston (13) laid special stress upon the fact that streptococci and staphylococci seem to be characteristic of sewage and animal waste, the former being, in his opinion, the more truly indicative of recent pollution. Horrocks (14) found these organisms in great abundance in sewage, and in waters known to be polluted but which contained no traces of *B. coli*. He found by experiment that *B. coli* gradually disappeared from many specimens of sewage kept in the dark at the temperature of an outside veranda. But the commonest forms which persisted were varieties of streptococci and staphylococci.

The first investigators in this country to call attention to these streptococci were Winslow and Miss Hunnewell (15) in 1901. They first observed them in the washings from the hands of school children, and have since isolated them from crude sewage and from many polluted waters. They found that in Smith fermentation tubes "dextrose-fermenting organisms, which the immediate inoculation of the dextrose broth showed to be present . . . in forty-eight samples, disappeared in forty-four cases during the two incubations of twenty-four hours each." On analysis, the persisting organisms were found to be streptococci or staphylococci which had overgrown or killed out the colon bacilli in nearly every case. In only three out of 157 samples of unpolluted waters, however, did they find streptococci when 1 c.c. of the water was examined.

A note by one of the present writers (16) in 1902 set forth the difficulty of making pure cultures of *B. coli* from inoculations of dextrose broth with fecal matter, if the incubation is carried too

far. It was found that other organisms, especially streptococci, develop abundantly and overgrow the colon bacilli, which are present in chief during the period of gas-formation.

Gage (17), at the Lawrence Experiment Station, has reported the streptococci present in the sewage of that city, while LeGros (18), in a recently published monograph describes many streptococci, all isolated either from the body or from sewage. It seems, indeed, from all existing evidence, that the streptococci are, as a class, closely associated with animal bodies, occurring either on the surface or within the intestinal tract. It seems reasonable, therefore, to regard these organisms as indicative of recent pollution, as suggested by Houston.

Since, as we now know, *B. coli* is widespread in nature, it may perhaps be present in considerable numbers in waters otherwise unobjectionable, although we have as yet no proof of this possibility. It is also conceivable that streptococci may be found in pure water, though all evidence at the present time seems to render this improbable. But it is at present very unlikely that both forms will occur abundantly at the same time in any unpolluted waters, while, on the other hand, we know that both are abundant in sewage. It will therefore obviously be of advantage in judging the quality of a water if the presence of both kinds of bacteria can be demonstrated in it.

With this end in view we have recognized the desirability of examining waters for both colon bacilli and streptococci, and have attempted to devise a method whereby this examination may be conducted with sufficient ease, quickness, and certainty to become a useful "laboratory test." For this purpose we have made use of some of the cultural reactions of the two organisms, especially those with dextrose and other sugar broths, and litmus lactose agar. Differences are also manifest in appearance, in gelatin and agar tube cultures, in the effect produced in nitrate and indol solutions, and on potato. A more comprehensive comparison of the two organisms may be made from the descriptions below:

CHARACTERISTICS OF COLON BACILLI AND SEWAGE STREPTOCOCCI.

COLON BACILLI.

SEWAGE STREPTOCOCCI.

FORM.

Bacillus 2-3 μ long by 0.5 μ wide; *Coccus* 1 μ in diameter.

Rounded ends.

GROUPING.

Occurs singly, or in short chains or masses. Occurs in short chains, often in pairs.

MOTILITY.

Actively motile.

Non-motile.

SPORE FORMATION.

None.

None.

GELATIN PLATE.

Thin, irregular film. Much as on agar, colonies smaller. Colonies similar to those on agar; no liquefaction.

GELATIN STICK.

Three days: transparent nail growth; gelatin not liquefied. Three days: nail growth, apparently made up of isolated colonies; very slight spreading on surface.

AGAR PLATE.

Twelve hours: surface colonies opalescent, nearly circular, edges smooth. Submerged colonies clear cut, lenticular. Two days: surface colonies take irregular vine-leaf form. Colonies small; under a low power somewhat irregular in shape; edges smooth.

AGAR SLOPE.

Twenty-four hours; luxuriant, moist, opalescent, translucent, white growth, narrowing from bottom to top. Twenty-four hours: faint dotted growth.

NUTRIENT BROTH.

Twelve hours: distinct turbidity. Eighteen hours: sediment. Two days: no scum on surface. Eighteen hours: turbidity and perceptible sediment; on shaking, sediment rises in spiral.

LITMUS MILK.

Litmus reddened, and then decolorized in twelve hours: milk coagulated in eighteen hours. Twelve hours: slightly acid; litmus slightly decolorized. Eighteen hours: strongly acid. Thirty-six hours: milk coagulated.

POTATO.

Luxuriant, dirty yellow growth; potato not discolored.	Hardly perceptible white growth after three days.
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DEXTROSE BROTH.

Twelve hours: dextrose fermented with formation of acid and gas.	Eighteen hours: strongly acid; no gas; sediment and turbidity in both arms.
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SACCHAROSE BROTH.

Twelve hours: saccharose fermented with formation of acid and some gas.	Eighteen hours: turbidity and sedi- ment, but no evidence of change in sugar.
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LACTOSE LITMUS AGAR PLATE.

Litmus reddened in twenty-four hours; bubbles of gas formed.	Twelve hours: litmus reddened; colo- nies small, with slight pink tint as if colored by litmus.
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PEPTONE AND SALT (DUNHAM).

Three days: pronounced nitroso- indol reaction.	Apparently no growth.
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ANAEROBIC AGAR SLOPE.

Thin, transparent growth, much less strong than in aerobic streak.	Dotted growth, less strong than in aerobic culture.
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Our preliminary experiments were based on the results of Winslow and Miss Hunnewell, and of one of the writers in the other papers mentioned. In view of our results, it seemed probable that there would be little difficulty in establishing a time relation between the growth periods of the two organisms in dextrose broth which would allow the isolation of both from the same culture if both were originally present in the sample used for inoculation. The soundness of this conclusion was first tested experimentally by the use of mixtures in known ratios of the two organisms in question, the culture used for this purpose being isolated from fresh feces by inoculation into dextrose broth, and plating out upon litmus lactose agar after six and forty-eight hours incubation at 37°C.

The method was as follows: Two bottles of sterile water, containing 100 c.c. each, were inoculated, one with *B. coli*, the other with streptococci, and plates made from these to determine the

number of each kind of bacteria used. With the water suspensions thus prepared mixtures were made, of various dilutions and proportions, of *B. coli* and streptococci. From each of these mixtures several Smith fermentation tubes containing dextrose broth (meat juice with 1 per cent. peptone and 2.5 per cent. dextrose) were inoculated with 1 c.c. each. The dextrose tubes were then incubated at 37° C., and litmus lactose agar plates made from them at various intervals. In plating, 1 c.c. of the dextrose broth culture was diluted in sterile water to approximately 1:100,000; 1:1,000,000; or 1:10,000,000; and 1 c.c. of this dilution inoculated into plates containing lactose agar to which sterile neutral litmus had been added. These plates were incubated at 37° C. for twenty-four hours, and then allowed to stand for some time at the room temperature before counting.

The colonies of *B. coli* and streptococci were distinguished by their different tints in the litmus lactose agar plate, and by further differences in shape and general appearance. The submerged colon colonies generally appeared of an oval or lenticular shape, and were not strongly acid. The streptococci, on the other hand, were most frequently spherical or nearly so, and became much more intensely reddened. After a little practice the two kinds of organisms could be detected without difficulty. In order to avoid error in this respect, however, cover-glass preparations from the separate colonies were examined microscopically for the morphology of the bacteria throughout the early part of the investigation.

The results obtained varied widely, as was to be expected, according to the number and proportions of the organisms inoculated. There was, however, in every case a period during which the colon bacilli were abundant in the dextrose tubes, while the streptococci were indistinguishable or nearly so. This condition was followed by a decrease in the number of *B. coli* and a sudden increase in streptococci, the colon bacilli at length becoming imperceptible and the streptococci rising to a maximum at which they persisted for a considerable time. The following tables show the numbers inoculated and the numbers found in the tubes at different platings:

TABLE I.

SERIES I.

	TUBE NUMBERS				
	1	2	3	4	5
Ratio—streptococci to B. coli inoculated.....	1:10	1:5	3:10	1:20	1:30
No. of B. coli inoculated....	145,000	145,000	145,000	290,000	435,000
No. of streptococci inocul't'd	14,500	29,000	43,500	14,500	14,500
Millions per c.c. of colon bacilli present after 9 hrs.	5.5	6.8	10	12	12.5
Streptoc'i pres'nt after 9 hrs.	0	0	0	0	0

NOTE.—This was a preliminary series to determine dilutions to be used and the general behavior of the bacteria. It was only possible to count the first plating.

The colonies of *B. coli* and streptococci were distinguished by the different tint in the litmus lactose agar plate, and by differences in shape and appearance. To be perfectly certain, however, cover-glass preparations from the separate colonies were examined microscopically.

SERIES II.

	TUBE NUMBERS				
	6	7	8	9	10
Ratio—streptococci to B. coli inoculated....	1:16	1:8	1:5	1:32	1:50
No. of B. coli inoculated....	100,000	100,000	100,000	200,000	300,000
No. of streptococci inocul't'd	6,000	12,000	18,000	6,000	6,000
No. found in tubes (millions per c.c.) after—					
18 hours, B. coli.....	600	500	440	420	410
Streptococci.....	0	0	0	0	0
27 hours ¹	400	600	500	450	300
42 hours, B. coli.....	0	0	0	...	0
Streptococci.....	62	120	135	...	154
51 hours, B. coli.....	0	0	0	0	0
Streptococci.....	60	100	115	90	85
65 hours, B. coli.....	0	0	0	0	0
Streptococci.....	70	65	35	80	80
95 hours, B. coli.....	0	0	0	0	0
Streptococci.....	90	70	48	85	90

¹ Proportions indeterminate.

SERIES III.

	TUBE NUMBERS		
	11	12	13
Ratio—streptococci to B. coli inoculated	21:1	210:1	105:1
No. of B. coli inoculated	2,250	225	450
No. of streptococci inoculated	47,000	47,000	47,000
No. found in tubes (millions per c.c.) after—			
9 hours, B. coli	Many ²	Many ²	Many ²
Streptococci	0	0	0
12 hours, B. coli	400	260	100
Streptococci	100	240	210
14 hours, B. coli	360	...	290
Streptococci	110	...	260
24 hours, B. coli	30	50	0
Streptococci	270	450	370
33 hours, B. coli	110	0	0
Streptococci	230	500	274
50 hours, B. coli	0	0	0
Streptococci	280	280	285
77 hours, B. coli	0	0	0
Streptococci	270	400	400

SERIES IV.

	TUBE NUMBERS		
	14	15	16
Ratio—streptococci to B. coli inoculated	1:100	1:1	100:1
No. of B. coli inoculated	160,000	1,600	1,600
No. of streptococci inoculated	1,700	1,700	170,000
No. found in tubes (millions per c.c.) after—			
11 hours, B. coli	350	320	300
Streptococci	0	0	0
14 hours, B. coli	640	350	200
Streptococci	30	70	170
19 hours, B. coli	400	340	32
Streptococci	110	104	124
24 hours, B. coli	494	354	74
Streptococci	80	96	284
33 hours, B. coli	346	112	0
Streptococci	100	58	280
41 hours, B. coli	3	0	0
Streptococci	60	83	147
67 hours, B. coli	0	0	0
Streptococci	6.2	0.18	0.51
85 hours, B. coli	0	0	...
Streptococci	0.22	0.22	...
Gas formation begun after	6 hours	9 hours	10 hours

² Count not made.

SERIES V.

	TUBE NUMBER	
	17	18
Ratio-streptococci to B. coli inoculated.....	1:2	53:1
No. of B. coli inoculated	32	3
No. of streptococci inoculated.....	16	160
No. in tube (in millions per c.c.) after—		
11 hours, B. coli	2.5	7.6
Streptococci	0.3	0.3
25 hours, B. coli	160	554
Streptococci	380	100
37 hours, B. coli	0.1	194
Streptococci	200	60
43 hours, B. coli	0	0
Streptococci	300	83
62 hours, B. coli	0	0
Streptococci	268	61
Gas formation begun after	15 hrs.	16 hrs.

To facilitate the systematic study of these results, we insert the following table, showing the time of approach of B. coli to a maximum, the time of appearance of streptococci, that of the approximate disappearance of B. coli, and that of the first formation of gas, in the few cases in which it was recorded.

TABLE II.

Tube Number	B. coli Inoculated	Streptococci Inoculated	First Gas Formed	B. coli Maximum	Appearance of Streptococci	Disappearance of B. coli
16.....	1,600	170,000	After 10 hrs.	After 11 hrs.	After 14 hrs.	After 33 hrs.
11.....	2 250	47,000	..	12	12	50
13.....	450	47,000	..	10	12	24
12.....	225	47,000	..	12	12	33
8.....	100,000	18,000	..	18	..	42
7.....	100,000	12,000	..	18	..	42
9.....	200,000	6,000	..	18	..	42
10.....	300,000	6,000	..	18	..	42
14.....	160,000	1,700	6	14	14	67
15.....	1,600	1,700	9	11	14	41
17.....	3	160	16	25	11	43
18.....	32	16	15	25	11	43

This table seems to indicate that, while the time of appearance of gas varies inversely with the numbers of B. coli originally present, the colon maximum and the appearance of streptococci

vary inversely with the number of streptococci first used, and the disappearance of the *B. coli* with the ratio of streptococci to colon bacilli. Some of the variations we are at a loss to explain, but it may be that further experiments will show that they were accidental.

EXAMINATION OF SEWAGE AND POLLUTED WATERS.

The results obtained with the mixture of known ratios gave promise of usefulness when the method was applied to actual examination of water and sewage, and in general the results have been much the same.

About fifty samples of waters have been examined. Each of these was tested quantitatively by making a suitable dilution, plating in litmus lactose agar, and incubating for twenty-four hours at 37°. The majority of the colonies thus obtained gave a well-marked acid reaction, but this was not specially recorded in the counting. At the time of plating 1 c.c. of each sample was inoculated into dextrose broth.

A preliminary series was first examined as a test of the method. The samples were as follows:

- a) Crude sewage from Dartmouth street (Boston) sewer, diluted 1:100, April 22.
- b) Charles River, at Otter street, April 22.
- c) Charles River, at Exeter street, April 22.
- d) Charles River, at Newton Lower Falls, April 30.
- e) Charles River, at Pinckney street, April 30.

The results obtained by plating after various periods are shown in the accompanying tables.

The method proving fairly satisfactory, a systematic examination of forty-six samples of water was next undertaken. The number of bacteria present was determined in some cases by plating on gelatin, and the number of acid-producing colonies in litmus lactose agar was also usually noted.

From the tables it is apparent that the waters showed in general the same periodic changes as did the artificial mixtures: pure cultures of *B. coli* were first obtained in the plates, then the gradual displacement of one form by the other took place, and

at length only streptococci or allied forms were discernible. In a few instances the results were somewhat obscured by the growth of a proteus-like organism which developed in the open arm of the dextrose broth tube, but in our opinion both *B. coli* and streptococci can be detected in water by this method in almost all cases when both organisms are present, provided the number of

TABLE III.

SERIES A.

	TUBE NUMBER		
	<i>a</i>	<i>b</i>	<i>c</i>
No. of bacteria inoculated	580,000	17,000	17,000
No. in tubes (millions per c.c.) after—			
11 hours, <i>B. coli</i>	274	100	280
Streptococci	0	0	0
14 hours	138	422
19 hours, <i>B. coli</i>	50	0	0
Streptococci	190	60	210
24 hours, <i>B. coli</i>	2	0	2
Streptococci	180	250	230
33 hours, <i>B. coli</i>	22	8	3
Streptococci	154	47	10
41 hours, <i>B. coli</i>	6	0	5
Streptococci	134	8	58
67 hours, <i>B. coli</i>	0	0	0
Streptococci	40	51	23
85 hours, <i>B. coli</i>	0	0	0
Streptococci	11	37	19
Gas formation begun after	6 hrs.	8 hrs.	8 hrs.

SERIES B.

	TUBE NUMBER	
	<i>d</i>	<i>e</i>
No. of bacteria inoculated	1,200	9,000
No. found in tubes (millions per c.c.) after—		
11 hours, <i>B. coli</i>	3	16
Streptococci	0	0
25 hours, <i>B. coli</i>	8	20
Streptococci	0	300
35 hours, <i>B. coli</i>	0
Streptococci	200
43 hours, <i>B. coli</i>	0	0
Streptococci	175	178
Gas formation begun after	13 hrs.	10 hrs.

conflicting organisms does not greatly exceed that of the colon bacilli.

That certain definite cultural relations exist between colon bacilli and streptococci when grown together in dextrose broth, and when no conflicting organism is present in great abundance,

TABLE IV.

SERIES I.

	SAMPLE NUMBER									
	1	2	3	4	5	6	7	8	9	10
Total bacteria, gelatin count.....	250	25	123	271	300	64	45	65	8	33
Acid-producing bacteria, litmus lactose agar....	4	10	9	5	8	55	35	460	1250	105
No. of bacteria (mill. per c.c.) after gr'th in dext. broth for—										
11 hours, B. coli.....	1	20	68.4	200	185	400	130	332	420	410
Streptococci.....	0	0	0	0	0	0	0	0	0	0
16 hours, B. coli.....	200	76	130	270	220	210	140	420	285	410
Streptococci.....	40	25	20	10	45	30	20	210	75	145
23 hours, B. coli.....	280	150	385	370	300	570	200	405	320	300
Streptococci.....	140	85	280	170	300	1700	110	350	370	350
39 hours, B. coli.....	0	0	25	110	0	210	20	24	105	...
Streptococci.....	474	420	480	300	390	170	400	105	250	...
63 hours, B. coli.....	0	0	0	0	0	12	8	0	0	0
Streptococci.....	2	0	0	45	1	2	45	150	86	170
First gas noted after (hrs.)	10	10	9	9	10	8	10	6	6	8

SERIES II.

	SAMPLE NUMBER						
	11	12	13	14	15	16	17
Gelatin count.....	20000	20000
Red colonies.....	0	6	0	0	0	1	8
No. of bacteria (mill. per c.c.) after gr'th in dext. broth for—							
8 hours, B. coli.....	1.2	11.4	6.8	6.7	6.9	8.5	0.5
Streptococci.....	0	0	0	0	0	0	0
22 hours, B. coli.....	300	500	350	450	440	358	2000
Streptococci.....	200	60	225	136	10	265	300
32 hours, B. coli.....	130	10	70	170	50	300	490
Streptococci.....	220	200	620	210	160	540	280
46 hours, B. coli.....	0	Few ³	0	100	100	Few ³
Streptococci.....	7200	Many	7000	3000	1000	Many
60 hours, B. coli.....	280	1	0	62
Streptococci.....	80	180	640	20

³ Count inaccurate, owing to presence of conflicting organisms.

is evident from the results with waters, as well as with mixtures of known ratio. The colon bacilli invariably develop with greater rapidity than the streptococci under the cultural conditions which we have employed, but the latter finally overgrow or kill out the colon bacilli. This fact is probably due to the greater sensitiveness of *B. coli* to the lactic acid produced in the medium by the streptococci over and above that formed by the colon bacilli themselves.

SERIES III.

	SAMPLE NUMBER							
	18	19	20	21	22	23	24	25
Gelatine count				Not made				
Red colonies	1	150	25	30	50	170	200	30
No. of bacteria (mill. per c.c.) after growth in dext. br. for—								
7 hours, <i>B. coli</i>	0.02	0.01	0.04	0.12	0.55	1.1
Streptococci	0	0	0	0	0	0.01
17 hours, <i>B. coli</i>	266	100	88	350	510	380	330	160
Streptococci	150	0	40	140	240	128	80	220
27 hours, <i>B. coli</i>	520	610	72	700	1000	740	100	300
Streptococci	800	860	670	1080	2500	4380	0	3900
40 hours, <i>B. coli</i>	0	0	10	22	36	7	7.2	...
Streptococci	252	330	260	22	66	60	5.2	...
52 hours, <i>B. coli</i>	10	16	38	20	70	35	10	27
Streptococci	40	16	3.8	31	41	25	10	30

SERIES IV.

	SAMPLE NUMBER	
	26	27
Gelatin count	1000	940
Red colonies	1200	20
Number of bacteria (millions per c.c.) after growth in dextrose broth for—		
6 hours, <i>B. coli</i>	{ Growths obscured by other organisms	
Streptococci		
19 hours, <i>B. coli</i>	250	230
Streptococci	52	300
31 hours, <i>B. coli</i>	180	120
Streptococci	440	300
43 hours, <i>B. coli</i>	90	480
Streptococci	400
54 hours, <i>B. coli</i>	12	15
Streptococci	470	620

Under the favorable conditions at first existing, the colon bacilli develop very rapidly, fermenting the dextrose with the formation of gas and acid in from six to eighteen hours (the time varying according to the number of these organisms originally present), and attain a maximum in from twenty to thirty-six hours. The streptococci gradually increase along with the formation of larger quantities of lactic acid, until at the end of fifteen to twenty-four hours they become distinguishable in plates inoculated from the dextrose tube. At about the same time the colon bacilli apparently become unfavorably affected by the increase in acidity, and

SERIES V.

	SAMPLE NUMBER								
	28	29	30	31	32	33	34	35	36
Gelatin count	5	41	34	45	160	214	...
Red colonies	Not determined								
No. of bacteria (mill. per c.c.) after gr th in dext. broth for—									
12 hours, B. coli	200	.. ⁴	.. ⁴	.. ⁴	120	120	3	50	300
Streptococci	0	0	0	4	20
24 hours, B. coli	100	380	370	410	400	330	...	80	360
Streptococci	900	60	380	620	500	140	...	150	180
36 hours, B. coli	290	50	80	20	50	20	440	20	60
Streptococci	50	150	440	280	340	1500	90	320	200
60 hours, B. coli	280	1	0	64	...	3
Streptococci	80	180	640	25	...	65

SERIES VI.

	SAMPLE NUMBER									
	37	38	39	40	41	42	43	44	45	46
Gelatin count	38	12	22	15	35	115	70	72	105	40000
Red colonies	16	2	2	2	31	6	13	16	15	1200
No. of bacteria (mill. per c.c.) after gr th in dext. broth for—										
12 hours, B. coli	55	16	22	0	70	100	300	700	130	... ⁵
Streptococci	0	0	0	0	0	0	0	0	10	...
24 hours, B. coli	360	Proteus	400	...	260	320	550	720	40	...
Streptococci	360			Few	140	170	300	500	200	...
36 hours, B. coli	0	0	70	25	.. ⁵	40	240	260	0	0
Streptococci	Many	500	300	75	...	100	100	180	Many	450
48 hours, B. coli	0	2	0	0	0	0	.. ⁵	0	0	0
Streptococci	2	6	3	40	1	26	...	9	10	7

⁴ Conflicting organisms.⁵ Other organisms partly obscured the medium.

rapidly decrease until they are no longer perceptible. The increase in streptococci is rapid after they appear—so rapid, in fact, that small numbers of *B. coli* would hardly affect the efficiency of an isolation made a few hours before the colon bacilli disappear. The streptococci attain a maximum soon after the other organisms disappear, and remain near this maximum for one or two days. The time of incubation allowable before making plate cultures for isolation of streptococci has, therefore, wide limits.

The time of beginning of gas formation is a factor of considerable importance. In the few cases in which this time was noted with our mixtures of known ratio, the colon bacilli attained their maximum numbers within a few hours after gas formation began. In the case of the polluted waters, however, a somewhat longer period may intervene between the first appearance of gas and the numerical colon maximum, but in our experiments the latter generally occurred within twenty-four hours of the time of inoculation. This fact has a direct bearing on laboratory procedure. Since, in ordinary practice with the fermentation tube as applied to water examination, gas formation is expected if *B. coli* is present, and since streptococci are likely to appear soon after gas formation begins, it would seem advisable to look for *B. coli* in the confirmatory tests immediately after the first appearance of gas, rather than after a twenty-four to forty-eight hour period, as has been customary in some laboratories.

From the results of this investigation we suggest as a method for the detection of *B. coli* and sewage streptococci in water the following procedure:

Inoculate the desired quantity of water, preferably 1 c.c., into dextrose broth, in a tube or apparatus suitable for the ready determination of gas production. Incubate at 37° C.

After a few (six to twelve) hours' incubation examine the cultures for gas. Within two or three hours after the gas formation is first evident plate from the broth, into litmus lactose agar, incubating for twelve to eighteen hours at 37° C. If at the end of this time no acid-producing colonies are present, it is probably safe to assume that there were no colon bacilli present. If, on the

other hand, red colonies are present, these must be further examined by the usual diagnostic tests for *B. coli*.

After the first plating from the dextrose broth replace the fermentation tube in the incubator, allow it to remain there for thirty-six hours, and then plate again in litmus lactose agar. This plating should give a nearly pure culture of streptococci, if these organisms were originally present in the water.

If colon bacilli are not found in the first set of plates, the streptococci may still be isolated, if present, by this method, or possibly after a somewhat shorter period of incubation than we have suggested.

A POSSIBLE RAPID TEST FOR STREPTOCOCCI.

Since the streptococci follow the colon bacilli in their development in the dextrose tube it is plain that they can easily withstand an amount of acid such as is produced in the tube by *B. coli*. We have taken advantage of this fact in attempting to devise a method for the detection of streptococci without waiting for colon bacilli to develop; since it seems desirable, if streptococci are to be reckoned with in the future as a part of the regular routine of bacteriological water analysis, to have this determination parallel with that of *B. coli*, and as rapid as possible. We have accordingly proposed to use for this purpose a medium of sufficiently high acidity to inhibit the growth of *B. coli* and of most water bacteria, but which shall still permit the development of streptococci.

The question, however, arises whether the desired result is to be determined by the kind of acid used or by the mere degree of acidity; *i. e.*, whether the effect is limited to the particular acid produced by colon bacilli, namely, lactic acid, or may be secured by the presence of a sufficient quantity of such acids as hydrochloric or sulphuric, as well as lactic. We have therefore determined by titration the approximate amount of acid produced in dextrose broth up to the period of maximum numbers of *B. coli*, and have prepared a dextrose broth of slightly greater acidity than that indicated by these titrations. On inoculation of such broth with fresh, raw sewage in great dilution, we have succeeded

in cultivating streptococci in a nearly pure condition after only 24 hours' incubation. Further consideration of this problem is reserved for another paper.

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THE TOXIC ACTION OF SCARLATINAL AND PNEUMONIC SERA ON PARAMÆCIA.*

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THE toxic effect of the blood serum of certain animals upon paramœcia was studied by Ledoux-Lebard.† He used the serum of the guinea pig, rabbit, goat, horse, sheep, beef, calf, white rat, pigeon, and goose, diluting with water at least twenty parts to one of serum, in order to render the solution isotonic with the paramœcia. Balbiani had already pointed out the fact that paramœcia would die in any serum which had not been diluted by more than an equal volume of water, this being due, not to substances in the serum toxic to paramœcia, but to its hypertonicity. According to Balbiani, paramœcia are isotonic with a 0.3 per cent. solution of common salt.

Ledoux-Lebard found that the blood serum of all the animals he tested had a more or less pronounced toxic effect upon paramœcia. This effect was shown, first, by a slowing of the movements; then, by an irregular, often rotating movement, which gradually ceased altogether, the paramœcia lying motionless at the bottom of the glass. Careful examination showed that this change in the activity of the paramœcia was due in the beginning

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to the formation of a long viscous thread at the caudal extremity, which impeded progression and caused the paramœcia to make irregular movements in the effort to get rid of it. This filament Ledoux-Lebard regards as a mass of excrement. In most instances the paramœcia became agglutinated by means of their filaments, but this was not invariably true, and was influenced by factors other than the presence of filaments and the slowing of the paramœcia, for it was absent in several cases when these two conditions were present. Ledoux-Lebard thinks that some reciprocal attraction must be present before agglutination can occur. After the paramœcia have become motionless, certain further changes occur, if the serum is toxic enough to cause their death. Their bodies become deformed, the contractile vesicles cease pulsating and dilate, the shape becomes oval, and death ensues. This was seldom earlier than after twenty-four hours. Agglutination occurred usually in two to three hours, slowing, in about fifteen minutes. In case all of the paramœcia did not die, the surviving ones were not found to have acquired immunity. The serum of guinea pig, white rat, goose, and cattle was found toxic in dilutions as high as 1:320; that of white rat and horse produced agglutination in the higher dilutions only. The serum of goose caused immobilization in a dilution of 1:320, more rapid in proportion to the dilution. Beef, rat, and goose serum had the highest degree of toxicity.

He also examined twenty-eight specimens of human serum, fourteen of which were from patients suffering from various diseases, fourteen from the freshly severed umbilical cord of normal parturient women. Six of the first fourteen were non-toxic; eight were toxic in dilutions of 1:20. Of the sera from the parturient women ten were very slightly toxic; four caused immobilization and death of almost all paramœcia within twenty-four hours. This variability was no greater than that seen in the individual cases among the lower animals, nor does Ledoux-Lebard seem to have found much difference between the normal and pathological sera. There are no details given as to the fourteen patients from whom the pathological sera were taken.

Exposure to a temperature of 55° C. for ten to thirty minutes

destroyed the toxic effect of rabbit and guinea-pig serum. Horse serum and beef serum required 60° C. The effort to restore this toxicity, by the addition of fresh, non-toxic human serum, failed.

These experiments of Ledoux-Lebard, suggesting as they do a new method of study of elements in the blood serum, seemed worthy, not only of confirmation, but of an attempt at further extension, especially into the domain of pathological human serum. Ledoux-Lebard's experiments in this line were not productive of positive results, yet it seemed possible that the examination of a larger number of specimens might show that the variations met with are not only those which are to be expected in different individuals, but that they are dependent upon the different diseased conditions.

The following studies were made upon the serum from cases of scarlet fever and lobar pneumonia chiefly, with a few cases of erysipelas and septicemia, of each of which only four or five specimens could be obtained.

The technic employed was very simple. Cultures of paramœcia in tap water were kept on hand, and the serum was added directly to this water in a dilution of 1:5, the mixture being made in an ordinary hollow-ground slide, which was then placed in a moist chamber. Ledoux-Lebard's dilution of 1:20 was used at first, but was abandoned for the 1:5 dilution, as this produces all the changes in the same order as the weaker, but much more rapidly. If a dilution of 1:20 is used, the first change, slowing and rotary motion on the part of the paramœcia, may occur almost as quickly as in the stronger solution, but very often this effect is transient, only a few paramœcia die, and the rest recover their active motility. This is what was found to occur usually with normal human serum, and often with pathological serum, which could not then be distinguished from the normal. If, however, a large proportion of serum was used, the difference became marked, for the pathological serum caused death within a few hours, while the normal caused only transient slowing, with perhaps the death of a few individuals.

The phenomena which occur in the paramœcia as the result of the toxic action of a serum were found to be the same as those

described by Ledoux-Lebard, with the exception of the agglutination, which was very rarely seen, probably because of the strong solution used. As already stated, Ledoux-Lebard failed to find it in the stronger solutions used by him, probably because immobilization and death occurred too soon. I could find no evidence of "reciprocal attraction." The agglutination, if present, seemed always due to the mechanical effect of the sticky threads attached to the caudal extremities of the paramœcia.

The first change observed as the result of the action of a toxic serum is that the paramœcium swims less rapidly, no longer darts across the field, but makes its way with some difficulty, and turns from side to side, now and then rotating rapidly for a few seconds, apparently in the effort to relieve itself of the clinging mass of excrement which hinders its movements. Even in this stage a dilation of the contractile vesicles may be observed, in cases where the serum is very toxic, but usually this does not appear until the paramœcia have become immobile and fallen to the bottom of the glass. Motionless individuals with some dilation of the vesicles may still recover, and after several hours may be found normal in appearance and activity. In other cases, if the serum is moderately toxic, they may die without further change; but if the serum is very toxic, they become greatly distorted in shape, the contractile vesicles dilate enormously, and coalesce into one large vacuole, which then forces its way to the surface, pushing the protoplasm to one side. Usually the outer covering is not ruptured, but the vacuole occupies the pointed end of the now pear-shaped paramœcium, the granular protoplasm filling the large, rounded end. The quicker the action of the serum, the greater the deformity produced. Paramœcia which die from starvation, from putrefaction in the culture medium, or from heat do not show deformity and seldom show dilation of the vesicles. In this connection may be mentioned an observation of Salomonson* on the negative chemotaxis exerted by dead paramœcia. This experimenter killed, by means of a hot needle, one or two individuals among a large number of paramœcia, and noticed that

* *Contributions to Celebrate the Inauguration of the State Serum Institute, Copenhagen, 1902.*

the living ones avoided these dead bodies, turning sharply away when they had approached within a certain distance. As I had never observed this avoidance of the dead bodies on the part of the living paramœcia in any of my examinations, I repeated Salomonsen's experiment with the hot needle, and found the result described by him, but found also that it was transient, lasting never more than fifteen minutes. After that the negative chemotaxis, whatever its nature, ceases to exert an influence, and the paramœcia swim indifferently around and over their dead comrades. It would seem that the effect must be produced by the hot needle, for it does not occur when paramœcia die from the effect of toxic substances.

A series of experiments as to the toxicity of the sera of different animals was first made, in confirmation of the experiments of Ledoux-Lebard. The effect in all these experiments is denoted toxic when all paramœcia are killed within twenty-four hours, incompletely toxic when some are still living at the end of this time, or when, although none have been killed, still the transient slowing and dilation have been observed. The degree of toxicity is shown by three asterisks when the full effect takes place within three hours' time, by two asterisks when it takes place in more than three and less than seven hours, and by one asterisk when it occurs between seven and eighteen hours. Incomplete toxicity is expressed by the combination of an asterisk and a line, and absence of toxicity by a line. The following results were obtained:

TABLE I.

The Toxic Action on Paramœcia of Serum of Lower Animals.

Rabbit	1	***	Beef	1	***
	3	***		2	***
	4	***		3	*** Serum
	5	***		3	* Blood
	6	***	Sheep	4	***
	7	***		1	**
	8	***		2	*
	9	***	Monkey		***
	10	***	Goat		<u>*</u>
Dog	1	***	Chicken		—
	2	*	Hog		—
	3	***	Guinea pig ...	1	***

It was found that the toxicity of blood was very much less than that of serum, and that serum left in contact with the clot sometimes showed an increase in toxicity after twenty-four hours. As a rule, the toxicity remained unchanged for the first two days, and then diminished progressively, disappearing between the third and fifth days. Heated to 55° C. for thirty minutes, all of these sera lost their toxic effect except the beef serum, in which case the heating had to be prolonged to one hour and a quarter before the toxic action disappeared completely.

The toxic substances are absorbed by the paramœcia, and, as shown by the following experiment, a serum can be rendered non-toxic by the addition of repeated doses of paramœcia:

TABLE II.

Exhaustion of the Toxicity of Serum.

Rabbit serum No. 8	-	-	-	-	1 gtt.
Paramœcium culture	-	-	-	-	3 gtt.
Death in thirty minutes.					
Paramœcium culture	-	-	-	-	1 gtt.
Death after eighteen hours.					
Paramœcium culture	-	-	-	-	1 gtt. (making the usual 1:5 dilution).
No effect after twenty-four hours.					
This serum was decanted off and fresh serum 1:5 added to the living paramœcia.					
Paramœcia died in forty minutes.					
The same results were obtained from beef serum.					

In order to determine whether the bodies of the dead paramœcia can take up this toxic substance, those killed in the last experiment were exposed to the action of fresh serum for an hour and a half, the serum then decanted and dropped upon living paramœcia. It was shown to have lost none of its toxicity; the bodies of paramœcia killed in this way cannot, therefore, absorb the active substance.

The sera of thirty-four adults in apparently normal condition were then examined (see Table III).

Thus among thirty-four normal sera nine were more or less toxic, three of these nine being markedly so without any apparent cause, as the individuals from whom the blood was taken were in perfect health. Those marked * caused a transient slowing and

swelling of the paramœcia, but no lasting effect. Serum No. 23 was examined four times, and only once exhibited any toxicity. Heating to 55° C. destroyed the toxic action in these nine sera.

One hundred specimens of serum from scarlet-fever patients were then examined, with the results shown in Table IV.

An analysis of these figures shows that of the one hundred sera eighty-five were toxic; the remaining fifteen were either non-toxic or only transiently or incompletely so. In one of these, blood was used instead of serum (203*b*), and, as had been found in the case of the serum of lower animals, the blood was almost inactive. Five of the fifteen non-toxic specimens of serum were

TABLE III.
The Toxic Action on Paramœcia of Normal Human Serum.

No. 1 ...	—	No. 10 ...	—	No. 19 ...	—	No. 27 ...	—
2 ...	**	11 ...	—	20 ...	—	28 ...	**
3 ...	*	12 ...	*	21 ...	—	29 ...	—
4 ...	***	13 ...	*	22 ...	—	30 ...	—
5 ...	—	14 ...	*	23 ...	***	31 ...	*
6 ...	—	15 ...	—	24 ...	**	32 ...	*
7 ...	—	16 ...	—	25 ...	—	33 ...	—
8 ...	—	17 ...	***	26 ...	*	34 ...	*
9 ...	—	18 ...	*				

taken comparatively late in the course of the disease (207, 196, 202, 199, 242), and in all but one of these the sera had proved toxic during the early stages of the disease. Another (206) was from a case in which there was great doubt as to the diagnosis, leaving eight non-toxic cases (204, 205*a*, 190, 220, 221, 262, 278, and 285*a*) which must be regarded as exceptional, as they were taken from cases of undoubted scarlet fever early in the course of the disease. Fifty-eight of the eighty-five toxic cases belonged to the group marked ***; that is, they produced the full effect, within three hours; fifteen were slightly less toxic, taking from three to seven hours to produce their effect; and twelve were still less toxic, taking from seven to eighteen hours. Of these twelve only five were sera taken early in the course of the disease; the remaining seven were either blood, not serum (229 and 230), or were taken late from cases in which the serum had formerly been very toxic (202, 230, 263, 272*b*, and 277*b*). As a rule, the blood

TABLE IV.
Paramœcidal Action of Scarlatinal Serum.

Case No.	Day of Disease	Degree of Toxicity of Serum	Character of Disease	Remarks
178.....	?	* * *	?	Ser. sent in from out-side hosp. No hist.
179.....	?	* * *	?	"
181.....	5th	* * *	Severe	
182.....	6th	* * *	Moderately severe	
184.....	4th	* * *	Mild	
185.....	5th	* * *	Severe	
186.....	?	* * *	Moderately severe	
188.....	?	* * *	?	"
190.....	4th	—	Severe	
192.....	3d	* * *	Mild	
193.....	13th	* * *	Moderately severe	
194.....	5th	* * *	Moderately severe	
195.....	3d	* * *	Moderately severe	
196 <i>a</i>	5th	* *	Mild	
196 <i>b</i>	6th	* *	Mild	
196 <i>c</i>	12th	—	Mild	
197.....	7th	*	Mild	
198.....	15th	* *	Moderately severe	
199 <i>a</i>	6th	* *	Moderately severe	
199 <i>b</i>	25th	—	Moderately severe	
200.....	4th	* *	Moderately severe	
201.....	7th	* * *	Mild	
202 <i>a</i>	7th	* * *	Moderately severe	
202 <i>b</i>	11th	* * *	Moderately severe	
202 <i>c</i>	13th	*	Moderately severe	
202 <i>d</i>	19th	—	Moderately severe	
203 <i>a</i>	7th	*	Moderately severe	
203 <i>b</i>	10th	*	Moderately severe	Blood
204.....	?	—	?	Ser. sent in from out-side hosp. No hist.
205 <i>a</i>	6th	*	Moderately severe	
205 <i>b</i>	16th	* * *	Moderately severe	
206.....	6th	*	Mild	Diagnosis doubtful
207 <i>a</i>	5th	*	Moderately severe	
207 <i>b</i>	25th	—	Moderately severe	
208 <i>a</i>	3d	* * *	Moderately severe	
208 <i>b</i>	10th	* * *	Moderately severe	
209 <i>a</i>	3d	* * *	Moderately severe	
209 <i>b</i>	10th	* * *	Moderately severe	
210 <i>a</i>	5th	* * *	Moderately severe	
210 <i>b</i>	15th	* * *	Moderately severe	
220.....	?	*	Mild	
221.....	5th	—	Moderately severe	
226.....	6th	*	Moderately severe	
229 <i>a</i>	3d	*	Mild	Blood
229 <i>b</i>	3d	* * *	Mild	
230 <i>a</i>	8th	* * *	Mild	
230 <i>b</i>	12th	*	Mild	Blood
230 <i>c</i>	12th	* * *	Mild	
230 <i>d</i>	13th	*	Mild	
231.....	3d	* *	Moderately severe	
234.....	2d	* *	Moderately severe	
235.....	2d	* *	Moderately severe	

TABLE IV.—*Continued*
 Paramœcidal Action of Scarlatinal Serum.

Case No.	Day of Disease	Degree of Toxicity of Serum	Character of Disease	Remarks
236.....	3d	* * *	Mild	
237.....	4th	* * *	Severe	
240.....	4th	* * *	Mild	
242.....	22d	—	Severe	Uremia
245.....	4th	* *	Severe	
249 <i>a</i>	4th	* * *	Moderately severe	Strep. in blood during life
249 <i>b</i>	9th	* * *	Moderately severe	
250.....	?	* * *	?	Ser. sent in from outside hosp. No hist.
251.....	4th	* * *	Mild	
252.....	3d	*	?	"
253 <i>a</i>	5th	* *	Moderately severe	
253 <i>b</i>	32d	* * *	Moderately severe	
254.....	5th	* * *	Mild	
255.....	7th	* * *	Mild	Diagnosis doubtful
256.....	4th	* * *	Severe	
257.....	?	* * *	?	
258.....	8th	* * *	Severe	
260.....	?	* * *	Moderately severe	
261.....	3d	* * *	Moderately severe	
262.....	3d	*	Mild	
263.....	21st	*	Mild	
267.....		* * *	Mild	
269.....	2d	* * *	Mild	
270 <i>a</i>	2d	* * *	Severe	
270 <i>b</i>	4th	* * *	Severe	
271 <i>a</i>	2d	* * *	Moderately severe	
271 <i>b</i>	7th	* *	Moderately severe	
272 <i>a</i>	15th	* * *	Severe	
272 <i>b</i>	24th	*	Severe	Blood taken 8hr. after death. Strep. in b.
273.....	3d	* * *	Mild	
274.....	3d	* * *	Moderately severe	
275.....	4th	* * *	Severe	
277 <i>a</i>	4th	* * *	Moderately severe	
277 <i>b</i>	17th	*	Moderately severe	
278.....	4th	*	Moderately severe	
280.....	3d	* * *	Mild	
283.....	5th	* * *	Very mild	
284 <i>a</i>	5th	* *	Moderately severe	Comp. with diph.
284 <i>b</i>	7th	* * *	Moderately severe	
285 <i>a</i>	3d	* *	Severe	
285 <i>b</i>	6th	—	Severe	
286 <i>a</i>	3d	* * *	Mild	
286 <i>b</i>	5th	* * *	Mild	Died on day b. was taken. Strep. in blood abundantly.
286 <i>c</i>	8th	* *	Mild	
288.....	3d	* * *	Moderately severe	
289 <i>a</i>	3d	* * *	Moderately severe	
289 <i>b</i>	5th	* * *	Moderately severe	
290.....	7th	* * *	Severe	

withdrawn early in the course of the disease was very toxic, and there was a gradual diminution of toxicity, with complete disappearance at varying periods, sometimes as early as the twelfth day (196), although a high degree of toxicity might persist up to the thirty-second day (253), or there might even be an increase, the second sample being more toxic than the first (253, 205).

It is easy to see from the table that the severity of the clinical symptoms had nothing to do with the toxicity of the serum, for a mild case sometimes yielded a serum much more toxic than that of a fatal case (compare 273 mild, with 285*b* fatal). Has the streptococcus anything to do with the toxicity of the serum? Paramœcia live and multiply in milk and bouillon cultures of streptococcus, as well as in sterile milk and bouillon; indeed, this organism seems to serve as food for them, for they multiply actively in hay infusion with which an agar culture of streptococcus has been mixed. Balbiani considers the ordinary bacteria of hay infusion the best nutrient for paramœcia, and apparently streptococcus can also be used as food, although putrefactive bacteria cause death of paramœcia in a few hours. Not only could no harmful action be laid to the streptococcus, but it was found that the growth of this organism actually diminished the toxicity of a normally toxic serum. Rabbit's blood serum has been shown to be toxic to paramœcia, and to remain so for twenty-four to forty-eight hours, even when kept at room temperature; but if this serum be inoculated with streptococcus and left for eighteen to twenty-four hours at room temperature, the toxic substances will have disappeared altogether. The same change takes place within the body of the animal. Two rabbits and one guinea pig were given intravenous injections of bouillon cultures of streptococci, isolated from the tonsils of three cases of scarlet fever. After the death of the animals the blood serum was found to be absolutely non-toxic. In the case of the rabbit and the guinea pig, therefore, it would seem that the substance toxic to paramœcia, far from being increased by the growth of the streptococcus, is usually destroyed by it. As for human serum, the presence of streptococci in the blood was demonstrated in cases 249, 272*b*, and 285*b*. The first of these sera was toxic in the highest degree, the second—a fatal

case—only slightly toxic, the third absolutely without effect. In these cases, therefore, it is impossible to attribute any action to the streptococcus. The sera seem to behave exactly as do the sera in which no organisms were found: 249, taken in the early stages was very toxic; 272*b*, in the latter, was less so; 285*b*, non-toxic on the sixth day of the disease, must be reckoned among the unexplained exceptions.

Three cases of septicemia, in two of which the streptococcus was grown from the blood during life, were tested, and also three cases of erysipelas, with the following results:

TABLE V.

	Erysipelas.					Puerperal Septicemia.				
1	-	-	-	-	-1	-	-	-	-	-
2	-	-	-	-	-2	-	-	-	-	*
3	-	-	-	-	*3	-	-	-	-	-

In other words, these six acted exactly like normal human serum, which is usually almost or quite non-toxic; and it is therefore impossible to say, as can be said of the normally paramœcidal sera of the rabbit and guinea pig cited above, that the streptococcal infection had destroyed the toxic substance.

The next question which suggested itself was whether the toxicity of scarlatinal sera would be affected by the streptococcus in the same way as is that of rabbit serum when inoculated with this organism. The sera from two cases of scarlet fever were inoculated with streptococci, and the cultures allowed to grow for twenty-four hours at room temperature.

EXHAUSTING TOXIC SUBSTANCES BY MEANS OF STREPTOCOCCUS.

Serum 195 (twenty-four hours old): paramœcia killed in twenty-four hours.

Serum 195, with twenty-four hours' streptococcus growth: no effect in twenty-four hours.

Serum 230*c* (twenty-four hours old): paramœcia killed in twenty-four hours.

Serum 230*c*, with twenty-four hours' streptococcus growth: no effect in twenty-four hours.

Apparently, then, the growth of the streptococcus in human serum outside of the body has the same effect as the growth in rabbit and guinea-pig serum within and without the body.

Of course it was possible that the effect produced on paramœcia by scarlet-fever serum might also be produced by the serum in other infectious diseases. As the sera from pneumonia patients were available in comparatively large numbers in the laboratory, these were chosen for purposes of comparison. Eighty specimens of serum from pneumonia patients were tested.

The toxicity of pneumonic serum varies greatly, but shows on the whole less divergence from normal serum than does scarlatinal, as can be seen by a comparison of the three:

TABLE VI.

	***	**	*	*	—
Scarlet fever.....	58	15	12	6	7
Pneumonia.....	26	12	8	19	15
Normal.....	3	3	3	6	19

Eighty-five per cent. of the scarlet-fever sera were toxic, 65 per cent. of the pneumonia, and 26.5 per cent. of the normal.

The toxicity of the serum in pneumonia bears no apparent relation to the stage of the disease or to the severity of the symptoms. The serum may be exceedingly toxic as late as the fourteenth day, or it may be non-toxic from the outstart, and this in cases rapidly fatal. The only thing which seemed to have a rather constant influence upon the serum in this respect was the presence of the pneumococcus in the blood. I am indebted for these details to Dr. Rosenow, who was at the time making a bacteriologic study of the blood of pneumonia, and who kindly gave me his results. It was found that only 26 per cent. of the blood sera which had proved toxic to paramœcia gave a fairly abundant growth of the pneumococcus, while 56 per cent. of the non-toxic contained abundant pneumococci.

These results can only be considered as suggesting the possibility that the toxicity for paramœcia of the blood serum in pneumonia is diminished or exhausted by the presence in the blood of large numbers of pneumococci, in the same way apparently as the normal toxicity of rabbit and beef serum and of scarlatinal serum is destroyed by the growth of the streptococcus.

The statement has already been made that the active substance in the blood sera of the lower animals which are toxic to paramœcia is destroyed by heating to 55° C., in some instances to 48° , and that it disappears spontaneously if the serum is left at room temperature for eighteen to twenty-four hours. This was found to be true of scarlet-fever serum also and of pneumonic serum; and further experiments served to show that the toxic substance is not simple, but is composed of at least two bodies, one of which is unstable, easily destroyed by heating to 48° or 55° C. (complement), and disappearing spontaneously after eighteen to thirty-six hours; the other stable, not easily destroyed by heat, and capable of uniting with a fresh complement to produce the toxic effect after the original complement has been destroyed.

Ledoux-Lebard suggested that this was the probable nature of the toxic principle in the blood serum of the lower animals, but he was not able to supply, with fresh non-toxic human serum, the complementary body of the serum of horse, guinea pig, rabbit, etc., which had been subjected to a heat of 55° to 60° C. for thirty minutes. It is possible, however, to reactivate the heated sera of these animals, with fresh serum of another species, and also to reactivate heated scarlatinal serum, although many attempts prove failures, and a fresh, non-toxic serum which serves to supply the complement to one heated serum often fails to do so for another of the same species.

The technic employed in reactivating serum was the following:

TABLE VII.

Paramœcia (ca. 200) in water	-	-	-	-	-	2.0 c.c.
Dog serum heated to 55° C. for thirty minutes						0.4 c.c.

Left over night in ice-box. Paramœcia lively next morning.

Poured into large vessel of tap-water and allowed to swim about for fifteen to thirty minutes. Fished out and placed in hollow-ground slide with normal human serum No. 6 (which had proved to be entirely non-toxic) in proportion of 1 part serum to 5 parts water. Paramœcia were slowed in thirty minutes. Dilation of vesicles and immobilization occurred in one hour and a half, death in two hours.

In the same way beef serum heated to 55° C. for one hour and a quarter was reactivated by three different specimens of fresh non-toxic human serum (Nos. 6, 8, and 11). The above experiment with dog serum heated to 55° and normal human serum No. 5 was repeated, but with this difference, that the human serum was three days old. The result here was negative, the three-day old serum failing to reactivate the heated serum.

The following experiment shows that low temperature temporarily suspends paramœcidal action:

TABLE VIII.

Fresh serum of dog	-	-	-	-	-	-	-	-	0.4 c.c.
Paramœcia in water	-	-	-	-	-	-	-	-	2.0 c.c.

Left overnight in ice-box. Paramœcia lively next morning, but on removing to room temperature they died in one and one-half hours. Repeated experiment with the same result.

REACTIVATION OF STREPTOCOCCUS SERUM.

a) Beef serum (streptococcus culture, eighteen hours)	-	1 part
Paramœcium culture	-	5 parts

No effect in two and one-half hours.

b) Beef serum (streptococcus culture, eighteen hours)	-	1 part
Paramœcium culture	-	10 parts
Normal human serum No. 6	-	1 part

Paramœcia showed slowing and swelling in thirty minutes, death in two and one-half hours.

REACTIVATION OF HEATED SCARLATINAL SERUM.

Serum No. 195 (heated to 55° C. for fifteen minutes)	-	0.4 c.c.
Paramœcium culture	-	2.0 c.c.

Left overnight. Paramœcia lively next morning. Poured into a dish of water and left for thirty minutes. Fished out and placed in hollow-ground slide with one part of fresh, normal human serum No. 12 to five parts of paramœcium culture. Death of all paramœcia in fourteen hours.

This experiment was repeated with this difference, that the normal serum was added directly to the mixture of heated scarlatinal serum and paramœcium culture with the addition of enough water to make the dilution the usual 1:5. The result here was the same as in the first experiment, and in the following experiments this simple technic was the one usually employed:

TABLE IX.

Reactivation of Heated Scarlatinal Serum.

Case No. of Scarlatinal Serum	Toxicity	Degree of Heat	Fresh Non-Toxic Serum	Result
201.....	* *	56° C.	10	Dead in eighteen hours
201.....	* *	56	206†	" " " "
202.....	* * *	56	10	" " " "
208.....	* * *	56	202d†	Dead in two and one-half hours
209.....	* * *	56	202d†	Dead in twenty-four hours
218.....	* * *	56	221†	" " " "
271 a.....	* * *	48	26	Dead in eighteen hours
272 a.....	* * *	48	28	" " " "
272 a.....	* * *	56	28	" " " "
272 a.....	* * *	60	28	" " " "
288 b.....	* * *	48	34	" " " "
288 b.....	* * *	56	34	" " " "
288 b.....	* * *	60	34	" " " "

† Non-toxic scarlatinal serum.

On the other hand, it was found impossible to restore the toxicity in a large number of cases. For instance, serum No. 99, which had restored the toxicity to No. 209, failed to do the same for Nos. 205 and 210; and there were many similar failures encountered in the effort to find suitable complements for the different sera.

The following experiment shows that the complementary body involved in producing the death of paramœcia is probably the same as that involved in hemolysis of rabbit corpuscles:

TABLE X.

Rabbit corpuscles washed and brought up to original quantity of	
blood by the addition of normal salt solution	2.0 c.c.
Serum	2.0 c.c.

added at once and then in smaller quantities until hemolysis seemed complete, the tube being kept at 37° C.

This serum, with its hemolytic complement for rabbit corpuscles thus exhausted, was added to paramœcia in the usual 1:5 dilution (allowance being made for the original dilution of the serum with normal salt), and it proved to have no effect on the paramœcia, its toxicity having entirely disappeared, while the control specimen of this same serum, No. 254, was toxic in two and one-half hours.

It has already been stated that the paramœcia which survived the action of a moderately toxic serum had not acquired any immunity thereby, but succumbed to a second dose of the same serum. Efforts were made to immunize the paramœcia against scarlatinal serum by adding repeated small doses of such serum to cultures of paramœcia at intervals of two to three days. The serum used in the first experiment was No. 202, and it was added in doses of 0.5 c.c. to 8.0 c.c. of the paramœcium culture which was kept in the ice-box. Three doses were given, at intervals of two days each. On the third day, after the last dose, the paramœcia were apparently perfectly normal. They were then treated with fresh serum No. 202, in the usual dilution of 1:5, and at the same time a control preparation was made with fresh paramœcium culture and serum No. 202. The paramœcia which had been treated with successive doses died in two and one-quarter hours, the control paramœcia a little later. The same negative results were obtained with serum No. 201. Heated serum was then used, first only three doses being given, then five, then seven; but in all these experiments the paramœcia thus treated showed

themselves, if anything, more susceptible to the usual dose of toxic serum than the untreated paramœcia.

The fact having been demonstrated that there are certain substances in the blood serum in scarlet fever toxic to paramœcia, the question then arose: Are those substances peculiar to the blood of this disease? Is there any qualitative difference between them and the toxic substances present in pneumonia and other diseases? Two lines of experiment were undertaken to answer this question.

In the first place, it seemed possible that there was a difference in the complementary bodies entering into the toxic principle in these two kinds of sera. As has been stated already, the complement in scarlatinal serum which is concerned in the destruction of paramœcia seems to be the same as that which is concerned in the hemolysis of rabbit corpuscles. A parallel experiment was made with pneumonic serum; the hemolytic complement for rabbit corpuscles was exhausted and the serum (formerly toxic to paramœcia) tested. In this case, just as in the case of the scarlatinal serum, the complement had been exhausted, and there was no toxic action on paramœcia, so that this attempt to prove a difference between the two bodies failed.

The second attempt was also a failure. It was thought that by heating the sera to different temperatures it might prove that the serum of scarlet fever could not be reactivated after a certain temperature had been reached, while the serum of pneumonia could still be reactivated, or *vice versa*. Experiments were made with eight scarlatinal and eight pneumonic sera. They were heated to 40°, 48°, 54°, and 60° C. The toxicity in all was destroyed at 48°, and the efforts to reactivate gave no results which pointed to any difference in the two kinds of serum. The reactivation, as always, was capricious: one eighteen-hour-old normal serum would supply the complement for a certain serum and not for others, but there was no apparent difference in the two kinds tested. Both scarlatinal and pneumonic sera can be reactivated after having been heated to 60° for thirty minutes, provided the right normal serum is found.

Mention has already been made of the fact that the blood of the lower animals proved to be less active than the serum. If the serum was removed from the clot immediately after coagulation and left on ice for twenty-four hours, it was found to be less toxic

than the same serum left in contact with the clot. The same was found to be true of the serum of scarlet fever and of pneumonia. The degree of toxicity of the blood never exceeded the lowest—death after seven hours—and in those cases where blood and serum from the same specimen were tested separately, the serum was always much more toxic (Case 229, third day, blood *, serum ***; Case 230, twelfth day, blood *, serum ***). Nor was the full toxic effect obtained if the blood was defibrinated and the serum separated by centrifugal action. Only when the serum was left on ice in contact with the clot for twelve to twenty-four hours was the full effect obtained, and in a few cases an even longer time seemed necessary. Three specimens of serum were most actively paramœcidal at the end of thirty-six hours.

These facts indicate that the leucocytes play an important part in the formation of the paramœcidal substance, and that this substance is probably liberated only after the disintegration of the leucocytes. Certainly such disintegration seems to increase the amount of toxic substance in the serum. The experiments in reactivation of heated sera, described above, tended to show that the element contributed by the leucocytes is the complementary body, for in these experiments it was found that normal human serum when perfectly fresh was not as rich in complement as the same serum kept on ice for twelve to eighteen hours. In some cases the older serum served to reactivate the heated serum when the fresh serum had failed; in others there was simply an increased activity in the older serum.

If, then, the leucocytes supply the complement which enables the toxic substance to attack the paramœcia, might there not be an explanation for the loss of toxicity observed in the sera inoculated with the streptococcus and in the cases of pneumonia which proved to have pneumococci in the blood? May not the streptococcus and the pneumococcus, as they develop, use up the leucocytic substances, and thus deprive the serum of its toxicity by depriving it of this particular elementary body? This theory would seem to be justified by the fact that loss of toxicity caused by the growth of the streptococcus is due to the loss of complement, as shown by the above-described experiments in the reactivation of such sera by fresh non-toxic serum.

CONCLUSIONS.

The serum of many animals (rabbit, guinea pig, dog, beef, sheep, monkey) is toxic to paramœcia.

Normal human serum is usually non-toxic, or very slightly toxic to paramœcia. Nine out of thirty-four specimens were more or less toxic.

The serum of scarlet fever is almost always toxic to paramœcia (85 per cent. of all cases examined).

The serum in pneumonia was toxic to paramœcia in 66 per cent. of all cases examined.

No qualitative differences in the toxic principle in the serum of scarlet fever and in that of pneumonia could be discovered.

The toxicity of scarlatinal serum is apparently independent of the streptococcal infection, being certainly not increased by the presence of the streptococcus in the blood during life, and decidedly diminished or destroyed by the growth of the streptococcus in the blood after death.

The toxicity of the serum in pneumonia is apparently diminished or destroyed by the presence in the blood of the pneumococcus in large numbers.

The paramœcidal substance in all these sera is composed of two different elements; the one being stable, resisting heat of 60° C. for thirty minutes or longer, persisting for at least three days in a serum kept at room temperature, and not affected by the action of the streptococcus within or without the body; the second being unstable, destroyed by heating to 60° C. for thirty minutes or more (in the case of scarlatinal and pneumonic sera to 48°), disappearing spontaneously after eighteen to forty hours at room temperature, and being exhausted or destroyed by the growth of the streptococcus.

The unstable complementary body can sometimes be supplied by the fresh serum of another species, normally non-toxic to paramœcia.

Experiments tend to show that the complement is chiefly or altogether contained in the bodies of the leucocytes, and exists free in the serum only after disintegration of the leucocytes.

PNEUMOCOCCAL BRONCHIOLITIS (CAPILLARY BRONCHITIS).*

C. P. CLARK AND F. H. BATMAN.

(From the Pathological Laboratory of Rush Medical College, Chicago.)

WHETHER capillary bronchitis constitutes a distinct affection is still a mooted question. Some authors hold that it is so closely related to tracheobronchitis or to bronchopneumonia that it should not be considered a separate disease, and others maintain that it is an established entity with a clinical as well as pathological basis.

Austin Flint¹ objected to the term because "the bronchial branches of small size, but not the smallest, are affected in so-called capillary bronchitis." Loomis and Thompson² maintain that "the capillary tubes are no longer regarded as the seat of a catarrhal inflammation which can with propriety be termed a capillary bronchitis as a pathological entity, since the process cannot extend to the terminal bronchioles without a corresponding lobular involvement." Many years ago Morrill³ remarked that the employment of the term "capillary bronchitis" to describe a distinct and independent disease had diminished, and that "the space allotted to it in the writings of the best authorities has been abridged." Christopher⁴ believes that this affection is always associated with bronchopneumonia, and that it does not exist as an independent affection. Hoffmann⁵ states that it is not separated from the simple acute tracheobronchitis by any sharp boundary line, and that the latter, if extensive, is always associated with the former. Osler⁶ considers the condition merely an early stage of bronchopneumonia, and he states that inflammation of the capillary bronchi rarely, if ever, exists without involvement of the lobular structure.

But opposite views are not wanting. Ewart⁷ believes that capillary bronchitis is a distinct disease, and that the occurrence of a bronchopneumonia is not inevitable, death occurring before

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consolidation appears, or the tendency being rather "to peribronchitis, to purulent infiltration of the bronchi, and to dilation, than to consolidation." Stewart and Gibson,⁸ J. M. Patton,⁹ Fowler and Godlee,¹⁰ Powell,¹¹ and Samuel West,¹² in recent publications, class this as a separate affection. According to West, capillary bronchitis has been established as a special disease both by clinical and pathological evidence, and it differs from bronchopneumonia in that the former is secondary to a tracheobronchitis, the latter being of acute onset without a previous bronchitis.

Doubtless the close relationship between this disease and bronchopneumonia accounts for the fact that the former resembles the latter in being primarily a disease of infants and the aged. Ewart states that it is "shared exclusively by the earliest and latest stages of life;" Fowler and Godlee, that when it occurs in mid-adult life it is among alcoholics and obese subjects.

Capillary bronchitis is considered by Duflocq and Ménétrier,¹³ Marfan,¹⁴ West, and others to be an infectious disease, and Duflocq¹⁵ and Ritchie¹⁶ report cases substantiating their opinion. Just as in tracheobronchitis, here also the most frequent organisms present are the pneumococcus and streptococcus.

That there may occur, in an adult, an inflammation, practically limited to small bronchi, is established by the following case.

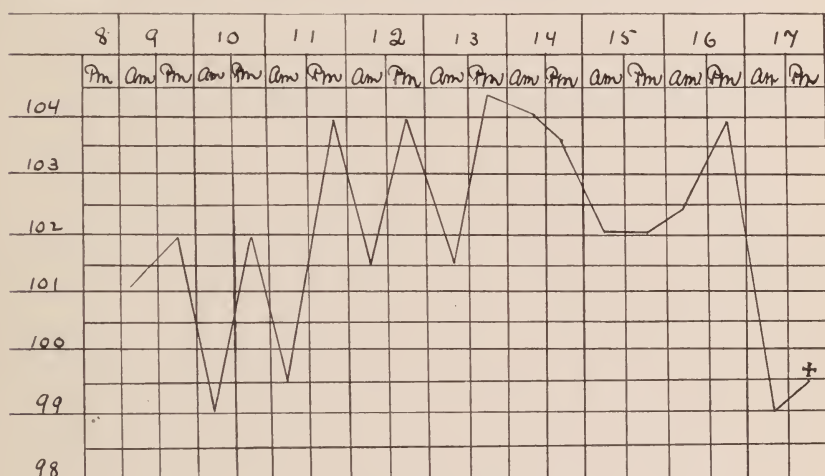
Abstract of the clinical record: Peter Damades, a Greek laborer, twenty-one years of age, was admitted to the Cook County Hospital to the service of Dr. E. F. Wells, April 8, 1903. The patient could not speak English, and the previous history was not obtained.

Examination showed a well-nourished young man, who perspired freely and coughed incessantly, raising a white expectoration. There was neither discomfort nor distress. The tongue was coated, and the pharynx, larynx, tonsils, and uvula were dusky red.

Externally the chest showed no changes, and the respiration was easy. Vocal fremitus was normal, and the movements on both sides were regular. Normal pulmonary resonance was obtained over the entire chest. Crepitant and subcrepitant rales

were heard below the third rib, anteriorly and posteriorly. The vocal sounds were unaltered, and tubular breathing was absent. The heart was normal in position, but the first sound at the apex was roughened. The spleen was not palpable. The sputum contained no tubercle bacilli, but diplococci and short bacilli. The urine was normal. There was a leucocytosis of 15,000.

The temperature on entrance was 101° F. The fever of onset was that of a gradual invasion, reaching a maximum height of 104.6° F. on the sixth day. Subsequently the temperature fell



TEMPERATURE CURVE.

and did not again reach its former height. It is noteworthy that during the last five days the course of the fever was in all respects similar to that of lobular pneumonia, the drop of 4.6° which occurred on the last day closely resembling a crisis (see curve).

The pulse and respiration were irregular, the former during the last few days ranging between 120 and 160; the latter, between 40 and 55.

On the tenth day after entrance the patient became delirious, ran into the corridor, and died soon afterward.*

The clinical diagnosis was pneumonia, probably associated with miliary tuberculosis.

The anatomical diagnosis (Dr. E. R. LeCount): bilateral dif-

*This is not an infrequent form of death in lobar pneumonia.

fuse bronchitis, bronchopneumonia (minimum degree), acute swelling of mediastinal and tracheobronchial glands, acute splenitis, hyperemia of the kidneys, hypertrophy of the heart, perisplenitis, miliary gummata (?) of the liver.

The great resemblance which the lesions of the lungs bore to tubercles was responsible for an anatomical diagnosis of general miliary tuberculosis of hematogenous type affecting both lungs. The subsequent histological examination, however, proved this diagnosis to be erroneous.

The main interest centers in the lungs, and the following brief description of them is taken from the record:

Combined weight of the lungs—1,780 g. The pleura of the right lung is glistening, and the margins are prominent. The lung is dark red, and the pleura quite free from coal pigment. The vessels are engorged. At one point externally there is a calcified area with a yellow center. On the posterior and diaphragmatic surfaces are found granular, projecting nodules. There are no adhesions between the lobes.

On the cut surface are seen disseminated white spots, not distinctly demarkated. These small pale areas of consolidation resemble miliary tubercles in all respects, except that they are not so sharply defined at the margins, coalescing gradually with the surrounding tissue. They are absent from the pleura, but are scattered throughout the lung quite uniformly, and in appearance are somewhat granular. One region of diffuse consolidation, as large as the finger nail, occurs in the upper lobe. It is granular and gray on the fresh surface. A yellow mucopurulent material exudes from the cut bronchi.

The left lung differs from the right only in the presence of a small amount of fibrous adhesion at the apex of the fissure, and a small area of atelectasis at the lower margin. Scattered throughout all the lobes granular areas similar to those in the right lung are found.

The tracheobronchial glands are enlarged. There is a large mass of them at the bifurcation of the trachea. They are soft, and the cut surface is moist and dark red. Several of the largest ones weigh together 200 g.

Bacterial examination.—The diplococcus lanceolatus was obtained in pure culture from the lungs and lymph glands. Cover-slip preparations showed this to be the sole organism present. It was encapsulated and stained by Gram's method. Cultures from the spleen and pericardial fluid remained sterile. A pure bouillon culture of the organism was injected into the peritoneal cavity of a rabbit. Death followed within thirty hours, and the diplococcus was recovered by cultural methods from the lungs, spleen, liver, heart's blood, and peritoneal fluid.

Histological examination.—Many of the bronchi (2–5 mm. in diameter) are normal. Others are filled with leucocytes and scattered patches of epithelial cells. In no instance does the wall show evidence of inflammatory change, hence it is likely that the leucocytes seen within the lumina have been derived from distant foci of inflammation.

The bronchioles of less than 1 mm. diameter are filled completely with polymorphonuclear leucotypes and desquamated epithelial cells. In some the epithelial lining is intact, but in the majority only patches of cells remain attached to the tunica propria, the detached cells lying within the lumen, often in long flakes. In others the epithelium is separated *en masse*, forming an isolated tube within the channel. Leucocytes are seen in great numbers between the cells of the attached epithelium. The tunica propria is edematous and greatly infiltrated with leucocytes. The capillaries are engorged, and in size resemble small veins. In many bronchioles these capillaries form the inner lining of the wall. The circular ring of muscle fibers of the bronchial wall is interrupted, and the muscle cells are seen in irregular patches lying among the other tissue elements. The perivascular lymph vessels are greatly dilated. The alveoli immediately surrounding the bronchioles are in many instances filled with leucocytes, epithelial cells, and red blood corpuscles. Those more distant are free from inflammatory products. The blood-vessels of the alveolar walls are intensely engorged.

In order to determine further the relation which the inflammation bears to the bronchioles, serial sections were made from several areas near the surface of the lungs. Sections, 10μ thick,

were cut, and every third was examined. The study of the series shows that the inflammation extends along the bronchioles and is for the most part confined to them, and the immediately adjacent alveoli, whereas distant alveoli are either unchanged or dilated.

From the foregoing description it is clear that the large bronchi are free from inflammation. The alveolar changes are slight considering the fact that the inflammation had existed for nine days, so that it is evident that the brouchopneumonia must be considered a secondary and not the primary lesion. It is readily seen that the bronchioles are the seat of the infection, and the case must be regarded as one of so-called capillary bronchitis, or preferable bronchiolitis. The inflammation was bilateral, affected all the bronchioles, and extended into the minutest subdivisions. The bacteriological examination showed the diplococcus lanceolatus to be the essential etiological factor—hence the disease must be termed a pneumococcal bronchiolitis.

There have been but few, if any, reports of similar cases with complete examination, but records of clinical cases have been published by P. Duffocq and others.

A point of interest in connection with this case is the fact that during the recent epidemic of lobar pneumonia in Chicago, within a period of six months, according to the report of the Board of Health, over three thousand individuals died from this disease; in the majority of these cases the organism produced, without doubt, a typical pneumonia, whereas in this case it produced a diffuse affection practically free from consolidation. The reason for this great difference is not clear, but perhaps it would be better understood if we knew more definitely the methods by which the pneumococcus attacks the lungs.

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IMPROVED TECHNIC OF AGGLUTINATION TEST IN TYPHOID FEVER—THE USE OF FORMALINIZED CULTURES.*

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AMONG the various methods employed in the serum test for typhoid fever two are especially popular and convenient of application. These are the dried-blood method, perfected and popularized by Wyatt Johnston, and the fresh-serum method, used by Widal. For laboratory and scientific purposes the fresh-serum method is preferable, as it offers a means of determining the maximum agglutinative power by making dilutions of various strengths. If the test is to be merely qualitative and for clinical purposes, and especially if the blood is sent by mail to a laboratory, the dried-blood method is by far the more convenient. Both methods are reliable and readily applied, but there is one drawback common to both. They both require a living culture of *B. typhosus* which should not be over twenty-four hours old, and this is not always obtainable except at institutions where the work is done on a large scale. Pröschner† has shown, however, that a twenty-four-hour bouillon culture of *B. typhosus* which has been killed with formalin gives the same specific reaction as the living culture when mixed with blood, or blood serum, from a typhoid-fever patient. It is this method which will be considered here.

Material for study was obtained from the cases of Dr. Billings and Dr. Herrick in the Presbyterian Hospital, and as typhoid fever was abundant during the summer and autumn of the year 1902, it afforded me an excellent opportunity to give the method a fair trial.

Technic.—About 500 c.c. of plain bouillon were inoculated with *B. typhosus*, incubated at 36° C. for twenty-four hours, and then the organisms were killed by adding 1 c.c. of formalin for every 100 c.c. of bouillon. A living twenty-four-hour bouillon culture was also kept on hand, and comparative

* Received for publication October 15, 1903.

† *Centralbl. f. Bakteriol.*, 1902, 31, p. 400.

tests were always carried out. The tests were made with fresh blood serum and with dried blood as follows: Five to 10 c.c. of blood were drawn from a superficial vein of the arm, allowed to clot in a sterile bottle, and placed in the refrigerator. The clear serum which exuded was taken from the clot after twelve to twenty-four hours and diluted with nine parts of distilled water, and the resulting mixture called a 1:10 dilution, each c.c. containing 0.1 c.c. of serum. Having made the 1:10 dilution of serum, small quantities were mixed with the living and also with the dead typhoid culture in the following manner:

1.0 c.c.	1:10 serum	to 4.0 c.c.	bouillon culture	= 1:50
1.0 "	"	"	5.0 "	" = 1:60
1.0 "	"	"	6.0 "	" = 1:70
1.0 "	"	"	7.0 "	" = 1:80
1.0 "	"	"	8.0 "	" = 1:90
1.0 "	"	"	9.0 "	" = 1:100
0.5 "	"	"	9.5 "	" = 1:200
0.25 "	"	"	9.75 "	" = 1:400

Now 1 c.c. of the 1:10 solution was mixed with 9 c.c. of distilled water, the dilution called 1:100, and the tests continued as follows:

1 c.c.	1:100 serum	to 4 c.c.	bouillon culture	= 1:500
1 "	"	"	5 "	" = 1:600
1 "	"	"	6 "	" = 1:700

These tests were continued until a dilution of 1:100,000 was reached to determine the least possible quantity of serum required to produce visible agglutination, and to compare the results obtained with the living and dead culture, also comparing the results obtained with blood serum and dried blood. A control tube, made by mixing 1 c.c. of a 1:10 dilution of normal blood serum with 4 c.c. of the typhoid culture, was always used.

In this manner thirty-four cases were studied, using fresh blood serum. At the same time tests were carried out by the dried-blood method, with both living and dead culture. Four drops of blood were spread on a glass slide, allowed to dry, and later dissolved in 2 c.c. of distilled water, making approximately a 1:10 dilution. One c.c. of this dilution was put into each of two test-tubes *a* and *b*. To Tube *a* were added 4 c.c. of the living culture, and to *b*, 4 c.c. of the dead culture. The tubes were now set aside together with those containing fresh serum, and the results compared (see Table of Results).

The lowest dilution at which the test was considered positive was 1:50. The second figures under each case indicate the highest dilutions at which visible agglutination took place inside of twelve hours.

Of the thirty-four cases studied thirty gave positive reaction, and confirmed the clinical diagnosis of typhoid fever which in the

TABLE OF RESULTS.

Case	Dead Bacilli	Living Bacilli	Remarks
1.....	1: 50 aggl. in 10 min. 1: 5,000 aggl. in 5 hrs.	1: 50 aggl. in 10 min. 1: 5,000 aggl. in 9 hrs.	Typhoid fever
2.....	1: 50 aggl. in 15 min. 1: 2,000 aggl. in 6 hrs.	1: 50 aggl. in 15 min. 1: 2,000 aggl. in 10 hrs.	Typhoid fever
3.....	1: 50 aggl. instantly 1: 8,000 aggl. in 5 hrs.	1: 50 aggl. in 5 min. 1: 8,000 aggl. in 8 hrs.	Typhoid fever
4.....	1: 50 aggl. in 30 min. 1: 600 aggl. in 7 hrs.	1: 50 aggl. in 40 min. 1: 600 aggl. in 12 hrs.	Typhoid fever
5.....	1: 50 no aggl.	1: 50 no aggl.	Doubtful
6.....	1: 50 aggl. in 30 min. 1: 1,000 aggl. in 8 hrs.	1: 50 aggl. in 40 min. 1: 1,000 aggl. in 12 hrs.	Typhoid fever
7.....	1: 50 aggl. in 20 min. 1: 2,000 aggl. in 6 hrs.	1: 50 aggl. in 30 min. 1: 2,000 aggl. in 10 hrs.	Typhoid fever
8.....	1: 50 aggl. in 30 min. 1: 5,000 aggl. in 5 hrs.	1: 50 aggl. in 35 min. 1: 5,000 aggl. in 8 hrs.	Typhoid fever
9.....	1: 50 aggl. in 30 min. 1: 6,000 aggl. in 5 hrs.	1: 50 aggl. in 40 min. 1: 6,000 aggl. in 7 hrs.	Typhoid fever
10.....	1: 50 no aggl.	1: 50 no aggl.	Paratyphoid
11.....	1: 50 aggl. in 20 min. 1: 1,000 aggl. in 6 hrs.	1: 50 aggl. in 30 min. 1: 1,000 aggl. in 8 hrs.	Typhoid fever
12.....	1: 50 aggl. in 5 min. 1: 2,000 aggl. in 6 hrs.	1: 50 aggl. in 10 min. 1: 2,000 aggl. in 10 hrs.	Typhoid fever
13.....	1: 50 aggl. in 30 min. 1: 1,000 aggl. in 5 hrs.	1: 50 aggl. in 40 min. 1: 1,000 aggl. in 8 hrs.	Typhoid fever
14.....	1: 50 aggl. in 20 min. 1: 2,000 aggl. in 5 hrs.	1: 30 aggl. in 30 min. 1: 2,000 aggl. in 9 hrs.	Typhoid fever
15.....	1: 50 aggl. in 10 min. 1: 4,000 aggl. in 6 hrs.	1: 50 aggl. in 15 min. 1: 4,000 aggl. in 8 hrs.	Typhoid fever
16.....	1: 50 aggl. in 20 min. 1: 500 aggl. in 6 hrs.	1: 50 aggl. in 30 min. 1: 500 aggl. in 8 hrs.	Typhoid fever
17.....	1: 50 no aggl.	1: 50 no aggl.	Paratyphoid
18.....	1: 50 aggl. in 5 min. 1: 20,000 aggl. in 10 hrs.	1: 50 aggl. in 5 min. 1: 20,000 aggl. in 12 hrs.	Typhoid fever
19.....	1: 50 aggl. in 40 min. 1: 500 aggl. in 5 hrs.	1: 50 aggl. in 1 hr. 1: 500 aggl. in 7 hrs.	Typhoid fever
20.....	1: 50 aggl. in 10 min. 1: 10,000 aggl. in 6 hrs.	1: 50 aggl. in 15 min. 1: 10,000 aggl. in 8 hrs.	Typhoid fever
21.....	1: 50 aggl. in 20 min. 1: 500 aggl. in 3 hrs.	1: 50 aggl. in 30 min. 1: 500 aggl. in 6 hrs.	Typhoid fever
22.....	1: 50 aggl. in 20 min. 1: 800 aggl. in 5 hrs.	1: 50 aggl. in 30 min. 1: 800 aggl. in 8 hrs.	Typhoid fever
23.....	1: 50 aggl. in 20 min. 1: 1,000 aggl. in 6 hrs.	1: 50 aggl. in 30 min. 1: 1,000 aggl. in 10 hrs.	Typhoid fever
24.....	1: 50 no aggl.	1: 50 no aggl.	Tuberculosis
25.....	1: 50 aggl. in 10 min. 1: 10,000 aggl. in 3 hrs.	1: 50 aggl. in 15 min. 1: 10,000 aggl. in 5 hrs.	Typhoid fever
26.....	1: 50 aggl. in 20 min. 1: 500 aggl. in 6 hrs.	1: 50 aggl. in 30 min. 1: 500 aggl. in 9 hrs.	Typhoid fever
27.....	1: 50 aggl. in 20 min. 1: 200 aggl. in 5 hrs.	1: 50 aggl. in 30 min. 1: 200 aggl. in 8 hrs.	Typhoid fever
28.....	1: 50 aggl. in 10 min. 1: 5,000 aggl. in 8 hrs.	1: 50 aggl. in 15 min. 1: 5,000 aggl. in 12 hrs.	Typhoid fever
29.....	1: 50 aggl. in 1 hr. 1: 100 aggl. in 6 hrs.	1: 50 aggl. in 1½ hrs. 1: 100 aggl. in 10 hrs.	Typhoid fever

TABLE OF RESULTS—*Continued.*

Case	Dead Bacilli	Living Bacilli	Remarks
30.. ...	1: 50 aggl. in 1 hr. 1: 80 aggl. in 5 hrs.	1: 50 aggl. in 1½ hrs. 1: 80 aggl. in 8 hrs.	Typhoid fever
31.....	1: 50 aggl. in 1 hr. 1: 100 aggl. in 6 hrs.	1: 50 aggl. in 1½ hrs. 1: 100 aggl. in 8 hrs.	Typhoid fever
32.....	1: 50 aggl. in 1 hr. 1: 100 aggl. in 5 hrs.	1: 50 aggl. in 2 hrs. 1: 100 aggl. in 7 hrs.	Typhoid fever
33.....	1: 50 aggl. in 40 min. 1: 1,000 aggl. in 5 hrs.	4: 50 aggl. in 1 hr. 1: 1,000 aggl. in 8 hrs.	Typhoid fever
34.....	1: 50 aggl. in 1 hr. 1: 200 aggl. in 5 hrs.	1: 50 aggl. in 2 hrs. 1: 200 aggl. in 9 hrs.	Typhoid fever

majority of these cases was established also by the isolation of the typhoid bacillus in cultures of the blood; two proved to be paratyphoid by bacteriologic examination of the blood; one doubtful; and the other tuberculosis.

In comparing the results obtained with dead cultures with those obtained with living cultures, it was found that the two differ very slightly. It was, however, noticed that the reaction becomes evident somewhat sooner with the dead than with the living cultures. This is especially noticeable in high dilutions which require some hours for the agglutination to become visible to the naked eye. Dried blood dissolved in distilled water gives practically the same results as fresh serum. Although the dried-blood method is very convenient for clinical purposes, one can save time and trouble by using fresh blood, which gives practically the same results as fresh serum or dried blood. Four drops of pure blood are allowed to flow into a small test-tube or vial containing 2 c.c. of a 1:500 solution of formalin in distilled water. Distilled water lyses the blood, forming a clear solution. The formalin is added to prevent bacterial growth in case some time elapses before the blood is tested. This gives approximately a 1:10 solution of fresh serum.

SUMMARY.

In summing up, briefly, the steps of the method most suitable for daily clinical use are as follows:

1. Inoculate a large quantity of plain bouillon (100–1,000 c.c.) with *B. typhosus*, incubate at 36° C. for twenty-four hours, and

add 1 c.c. formalin for every 100 c.c. of bouillon. The culture is now ready for future use, except that it must be shaken before it is used, because the dead organisms gradually settle to the bottom. A culture prepared in this manner is always ready for use, and can be kept at room temperature for many months.*

2. With a lancet or some other sharp instrument prick the lobe of the ear or the finger tip (preferably the lobe of the ear) and collect four drops of blood in a small test-tube or vial containing 2 c.c. of a 1:500 solution of formalin in distilled water. Laking is soon complete, making a clear solution of approximately 1:10.

3. To 1 c.c. of the blood solution add 4 c.c. of the dead culture, making a dilution of about 1:50, and set the tube aside.

4. Make a control tube by mixing 1 c.c. of a 1:10 solution of normal blood, or 1 c.c. of distilled water, with 4 c.c. of the culture, and set it aside.

If agglutination takes place, the dead organisms collect in clumps, and within an hour or two are seen as a flocculent precipitate settling toward the bottom, leaving the bouillon clear at the end of twelve to twenty-four hours.

Dried blood may be used instead of fresh blood. On a glass slide collect four drops of blood, spread it, and allow it to dry. When wanted for use, dissolve it in 2 c.c. of distilled water and proceed as directed above.

Among some of the general deductions from this and other recent work on the serum test in typhoid fever, the following seem worthy of special stress: By keeping on hand and using typhoid cultures killed by formalin, one is always prepared to apply the serum test without the use of a microscope or other apparatus outside of one or two small test-tubes or bottles. The time consumed is practically nothing, and the method appears to be suitable for hospitals, boards of health, and private practice.

*On August 20, 1903, a good reaction was obtained with a culture killed August 20, 1902—that is, one year after having been killed.

BACILLUS MUCOSUS CAPSULATUS.
A STUDY OF THE GROUP AND AN ATTEMPT AT CLASSIFI-
CATION OF THE VARIETIES DESCRIBED.*

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SINCE Friedländer, in 1883, first called attention to the presence of capsulated bacilli in pneumonia, a number of observers have described organisms more or less similar in form, and found in a variety of pathological lesions. The tendency to describe bacteria as new, if they differed in any way from previously described organisms, has been carried to its widest limits in this group. Fricke, in his exhaustive monograph, has analyzed twenty-two varieties described under different names, and to these must be added two others, noted by observers in this country. All these organisms were conceded to have certain main characteristics in common; but inasmuch as they did not in all respects agree with their prototype, the pneumo-bacillus of Friedländer, they were described in detail, and given a number of names.

Of late the work of bacteriologists has tended rather to the grouping of closely allied bacteria as sub-varieties under a single head than to multiplying varieties on the ground of minute and often inconstant characteristics. The almost infinite capacity for variation in the appearance of sub-cultures from the same colony, if the circumstances of growth are changed even to a minor degree, is a clearly sufficient basis for such a tendency. The necessity for classification of species on permanent and universally found differences is something that need not be dwelt on, and the simpler such classification can be, coincident with clearness, the more valuable it will be, not only to the specialist along these lines, but to the more or less casual worker who is at present often lost among the multiplicity of detail.

* Received for publication December 1, 1903.

In three years' work as resident pathologist of the Lakeside Hospital, the writer was struck with the frequency of bacteria, apparently of the *B. mucosus capsulatus* group, in the routine autopsy plates. These were made at all autopsies from the heart's blood, the lungs, the liver, the spleen, and the kidneys, and from any other organs or fluids which showed changes indicating the possible presence of micro-organisms. In about one-fourth of all cases colonies of the *B. mucosus capsulatus* type appeared on the plates in sufficient numbers and in such varied distribution as to exclude accidental contamination. Plates were, however, made as a check, from the air of the different rooms where the bacteriological work was done, but in no case was an organism of this type recovered.

A piece of work was begun with the bacteria isolated, but, owing to certain accidents, these were lost, and the research remained incomplete. Enough has been recorded to establish the relationship of these earlier cultivated organisms with the later ones, and with the group under consideration.

According to Fricke, the general characteristics of the group are as follows: The organisms are short, possess capsules, show marked pleomorphism, and form no spores. They are not motile, and decolorize by Gram's method. They grow on a variety of media in a profuse slimy layer, and in gelatin slabs they do not liquefy, but show the so-called "nail-growth." In addition to this, most of the bacteria classed under this head form a moderate amount of indol, and ferment carbohydrates in solution with the formation of gas and acid.

In 286 consecutive autopsies in which routine cultures were made, organisms answering to the above description were isolated in 79 cases. In all but 5 of these the cultures came under my personal observation, and in the others the recorded descriptions are not sufficiently worked out to exclude the colon bacillus, and will therefore not be considered. This leaves 74 cases, or 25.8 per cent. of cases studied bacteriologically. The bodies of persons dying in the hospital are placed at once in cold storage at 0° C., and autopsies are almost always performed within twenty-four hours of death.

DISTRIBUTION.

The occurrence of *B. mucosus capsulatus* has been noted by many authors, and in a great variety of places and lesions. The literature in this respect has been so fully covered by Fricke in 1896, and Clairmont in 1902, that it seems scarcely worth while to do more than summarize the findings.

Outside of the animal body it has been found in the soil, in the air, in cracks in the floor, in dust, and in water. It has been found in human beings in health, in the nose frequently, in the mouth more rarely (according to Netter in 4.5 per cent. of cases), in saliva, and in the gastro-intestinal tract. In disease it has been isolated from the sputum of influenza patients, and of persons with pneumonia and tuberculosis, of course in association with other organisms. The pathological lesions in which it has been found may be divided according to their relations to the body functions:

In the *respiratory tract*, it has been found in rhinoscleroma, ozena, inflammatory conditions of the nasal passages and accessory nasal sinuses, in lobar and bronchopneumonia, in abscess and gangrene of the lungs, in bronchitis, and in bronchiectasis; in the *digestive tract*, in stomatitis, gastroenteritis, dysentery, and appendicitis; in *serous cavities*, in pleurisy of various types, in pericarditis, and in peritonitis, usually perforative in origin; in the *urinary tract*, in cystitis, acute and chronic, pyoureter, pyonephrosis, pyelonephritis, abscess of the kidney, and in infected adenocystoma of the kidney; in the *circulatory system*, in acute ulcerative endocarditis; in the *genital tract*, in acute endometritis; in the *nervous system*, in brain abscess, gas cysts of the brain, meningitis; in the *special sense organs*, in otitis media.

Besides these local infections, a number of cases have been described in which there has been a *general infection*, often of the hemorrhagic type, as noted by Howard, Blumer and others.

Distribution in Cleveland.—A number of attempts were made to ascertain the distribution in Cleveland. Plates from earth obtained along the streets in different parts of the city showed no organisms of this type. Investigations pursued independently by the class in bacteriology, and by the City Bacteriological Labora-

tory, failed to show it in city water. (Since the writing of this article, organisms of the group have been found in the city water supply, 1903.) Air plates in the laboratories of the Medical School, and those of Lakeside Hospital, were also negative in this regard. A series of tests by the City Laboratory in 1902 (repeated in 1903), showed none of this group in a large series of vaccine points and tubes from different makers.

In eighteen plates made from the throats of members of the class in bacteriology, all of whom considered themselves well at the time, only one showed cultures of the desired kind.

At eighteen successive autopsies at Lakeside, cultures were made from the stomach, small and large intestine, before these were opened, in the usual manner. In ten of these it was possible to isolate organisms of this group, the so-called "aërogenes," from small and large intestines, but in no case from the stomach. It is probable that the presence of the colon bacillus may in some of the other cases have obscured the colonies of *B. mucosus capsulatus*, so that the number of positive cases is perhaps larger.

The series of seventy-four cases from the Lakeside Hospital covers quite a wide pathological field, and the lesions in which the organisms were found may be summarized as follows:

Bronchopneumonia - - -	19	Adenocystoma of kidney infected	1
Lobar pneumonia - - -	1	Tubercular abscess of kidney -	3
Hypostatic pneumonia - - -	1	Peritonitis, purulent - - -	6
Bronchitis - - - - -	5	Pericarditis, purulent - - -	1
Gangrene of lung - - - -	1	Endometritis, acute - - - -	1
Lung tuberculosis, cavity - -	3	Omphalitis, acute - - - -	1
Miliary tuberculosis of lung -	1	Otitis media - - - - -	1
Infarction of lung - - - -	1	Carcinoma of liver - - - -	1
Hemorrhage into lung - - -	1	Gas cysts of brain, general gas-	
Cystitis - - - - -	3	eous emphysema - - - -	1
Pyonephrosis - - - - -	2		
Pyelonephritis - - - - -	2	Total - - - - -	56
Pyoureter - - - - -	1		

In 14 cases the organisms were found distributed through all organs, though none could be classed as hemorrhagic septicemias, the nearest approach being the one in which gas cysts of the brain and a general gaseous emphysema were observed. In the

other cases there were no lesions found to account for the presence of the organisms.

In hospital cases outside the autopsy service, bacteria of this type were found in three appendix cases in the surgical service, and in a small pustule on the face of one of the laboratory attendants. The organism belonging to this group which caused a gaseous and hemorrhagic epidemic among the laboratory guinea pigs has already been noted elsewhere, and comes under the first class in the final classification in this paper.

In the present piece of work, 37 organisms were studied, 19 of them being from the Lakeside material, and the other 18 from various sources, with the idea of comparing as many members of the group as possible under the same cultural conditions.

From Král's laboratory the following strains were secured: *B. pneumoniae* Friedländer, *B. capsulatus mucosus* (Kruse, Pauling, Fasching), *B. aërogenes* Escherich, *B. acidi lactici*, *B. crassus sputigenus* Kreibohm, *B. pseudo-pneumonicus* Passet, *B. capsulatus* Pfeiffer, *B. ozænæ* Abel, *B. rhinoscleromæ*; through the courtesy of Dr. Harris, of the Johns Hopkins Hospital, six strains isolated from various sources and classified as *B. pneum.* Friedländer; through the courtesy of Dr. Blumer, of Albany, *B. mucosus capsulatus* Blumer, described by him in a case of hemorrhagic septicemia; and from the stock cultures in my laboratory, *B. hemorrhagic septicemia* Howard, and *B. mucosus capsulatus* Wright and Mallory.

GENERAL TECHNIC.

The series was inoculated into plain, glycerin and glucose agar, gelatin, milk, dextrose-free and ordinary bouillon, potato, blood serum, and dextrose-free bouillon containing 1 per cent. of the various carbohydrates under consideration. Where possible, the media were made up according to the specifications of the American Public Health Association, and titrated to a reaction of + 1.5 to phenolphthalein.

The appearances on the different media will be taken up separately, with discussion of the results of other observers. In the tabulation it will be noticed that only twelve of the Cleveland cultures are analyzed. The other seven were so exactly like some of

those tabulated that it would be a useless repetition to set them down in full. B. m. c. 13, from kidney Aut. 238, was identical with B. m. c. 1; the kidney showed no lesion. B. m. c. 14, 15, 16, 17 were identical with B. m. c. 3; they were all from the lungs, which showed no pneumonia, but congestion and edema. B. m. c. 18, 19, were from the liver in one case and the spleen in another, and identical with B. m. c. 5; neither organ showed marked change.

GROWTH ON DIFFERENT MEDIA.

Plain agar.—The colony appearance on this medium was studied with a view of ascertaining whether the differences in the colonies of the different varieties were as constant and valuable as many writers state them to be. Strong has formed two main groups:

I. Young colonies colorless, older whitish, capsules present only in animal exudates and easily stained. In this class he places *B. pneumoniae* Friedländer, Wright and Mallory, *B. ozænae* Abel, *B. crassus sputigenus*, and perhaps rhinoscleroma.

II. Colonies white from the first, capsules hard to demonstrate. In this class come *B. aërogenes*, Pfeiffer, Kruse.

In plates from all the Cleveland series, except No. 6, the colonies were in general of the second type. In the cultures obtained elsewhere, *B. aërogenes*, *B. capsulatus septicus*, *B. pseudo-pneumonicus*, Fasching, Pfeiffer, Howard, and Blumer also came under this head.

Rhinoscleroma, and Wright and Mallory, were still pale at thirty-six hours, but Friedländer, *B. ozænae* Abel, and *B. sputigenus crassus*, though pale at the very first, after twenty-four hours showed no essential difference from those of the second group.

It was very noticeable that colonies on the same plate often differed widely in appearance, both as to color and as to appearance under the low power. Further development of such colonies showed them to be similar, and plates made from any single colony showed the same variations. When the colony started just below the surface and broke through, or when the agar was a little dry, the tendency seemed to be toward the whiter color.

In general, the colonies were raised, white, moist, glistening, usually round, but at times with wavy borders, and were yellowish by transmitted light. Under the low power they were finely to coarsely granular, the coarser granules being usually at the center and shading off at the edges to an almost transparent border. No absolute essential and differentiating characteristics could be seen, agreeing with the observations of Fricke, Clairmont, and others. In my work the divisions made by Strong did not appear to be constant.

Glycerin agar slants.—The growths, while very characteristic of the group as a whole, showed no constant differences. In the majority of cases the growth was profuse, much wider at the bottom than at the top, moist and shiny, with a well-marked porcelain-like appearance. The edges were at times sharply defined, and at times shaded off into the media. The center line was sometimes raised, sometimes in the form of a shallow groove. The stab was granular, showed good growth all the way down, and in most cases there was profuse development of gas. The water of condensation was heavily clouded, and there was usually a large flocculent sediment. The viscosity varied, being absolutely constant only in *B. rhinoscleromæ* and in No. 9 of my series.

The growth of *B. rhinoscleromæ* was rather thin and paler than the rest, the water of condensation was always very viscid, and no gas was formed in the stab.

In general, the less moisture in the agar, the greater the tendency to a raised, sharply defined, rather narrow growth, which in media containing more water tends to spread out toward the sides and to be thinner, especially at the edges. The gas formation is much more extensive in fresh moist agar.

Glucose agar.—The growth on this medium showed nothing additional to the above, except that the organisms capable of fermenting gave more gas than in glycerin agar.

Löffler's blood serum.—The cultures on this medium were not characteristic. The growths were profuse, yellowish white, and could not be told apart when the labels were covered. No liquefaction ever took place and no especial odor was ever noted.

Gelatin.—Several series of tubes were inoculated at different times during the year, and observed over a period of four weeks each. They were kept at room temperature, and exposed to ordinary daylight, though not to direct sunlight. One series was made on media a week old; the others, on media within twenty-four hours from the last sterilization.

In the first series, where the media were a little more dry, the characteristic round, elevated, so-called "nail-culture" was more frequent than in the subsequent inoculations, suggesting that the amount of moisture in the gelatin may have a very definite influence over that phenomenon. Pfeiffer's bacillus, which, according to Clairmont, never forms the nail-growth, did form it in the dryer gelatin, though not in the other.

In general, in the 38 cultures examined, 15 had rounded tops in all inoculations, while 5 always grew in a flattened mass on the surface, with no suggestion of a nail-head. The other 18 varied in different series, never being very typical.

In the stab, 18 were very coarsely granular, without any branches projecting from the sides, while 8 were always finely granular, usually with fine feathery outgrowths. The rest were sometimes finely, sometimes coarsely, granular. The feathery outgrowths in the stem were constant in 16 and absent in 22. The irregularity of these appearances and their relations to the different organisms indicate that there can be no absolute dependence placed on the gelatin growth. Development of a brownish color in the media after long periods of growth—a phenomenon also observed in glycerin agar—was in both media variable and inconstant. The cloudiness just below the surface, observed by Herla, Wilde, and Scheffer in old cultures, but never seen by Clairmont, was quite frequent, but not constant in any case. When the gelatin was made from dextrose-free bouillon, no development of gas was ever seen.

Milk.—Inoculations were made into litmus milk several times, at wide intervals, and organisms that were passed through animals were tested again after recultivation, to ascertain if there were any changes.

Although there was a moderate variation in the time of coagu-

lation of different samples of milk, and although the amount of acidity varied somewhat, still those which coagulated in the first instance always coagulated, and those which did not coagulate in the first instance never did so. To this there was one exception, *B. pseudo-pneumonicus* Passet. This, when received from Král, neither coagulated milk nor fermented sugars, but after passage through animals was able to do both. The other organisms which, when received, did not ferment milk, were also passed through animals, but neither this procedure nor prolonged cultivation in milk made any difference in their powers.

The statement widely made, both in the original article and in a variety of text-books, that Friedländer's bacillus ferments lactose, but does not coagulate milk, is denied by Strong. Clairmont finds traces of gas formation from lactose, and no coagulation of milk, while Fricke finds both gas formation from lactose and coagulation of milk. As to actual gas formation, it was noted that, unless the sample of lactose from which the dextrose-free bouillon was prepared was chemically pure, traces of gas might be found. There is also a possibility that the bouillon was not completely dextrose-free. In all our trials of the organisms which were included in this type (Class II in the final classification) we never found any gas formation from lactose, nor any coagulation of milk. The reaction was, however, as noted below, slightly acid, and in the fermentation tubes there was a very slight cloud in the first twenty-four hours, which usually disappeared before the forty-eight-hour examination. It is therefore possible that there may be a very slight fermentation for lactose, though it seems more probable that in the case of the milk the acid reaction is due to the presence of some dextrose, and that traces were also present in the fermentation tube.

The average time of coagulation varied somewhat, most of the lactose fermenters causing a firm clot in from twenty-four to forty-eight hours. Pfeiffer's bacillus, as seems to be the observation of most writers, takes from forty-eight to seventy-two hours, and in the present series Nos. 5, 10-12, and Howard, showed the same characteristic.

Those which did not ferment lactose were observed with especial care and frequency, and at no time was there any coagu-

lation, though in many cases a small amount of acid formation was seen. Wright and Mallory, said in the original to coagulate slowly, gave acid, but no coagulation, as was also observed by Strong. No. 6, and *B. sputigenus crassus* also gave an acid reaction. Hopkins cultures Nos. 3, 4, and 6, and Friedländer, were amphoteric. Fasching and *B. rhinoscleromæ* were persistently alkaline.

Howard found that Pfeiffer, Howard, and a bacillus from the antrum coagulated in from forty-eight to seventy-two hours, Wright and Mallory in from twenty-four to forty-eight, and that a culture from Reed of Washington, made from the throat in a case of diphtheria, as well as the laboratory Friedländer, did not coagulate at all.

Examination of the recorded cases at Lakeside Hospital shows that of the seventy-four recorded cases, only four did not coagulate. One of these, re-examined in the present series, No. 1, does coagulate in from twenty-four to forty-eight hours, indicating that in the whole series not more than four can be said to belong to the type characteristic of Friedländer.

Clairmont divides his organisms into three groups according to the time of coagulation, but, in view of the variation found in different samples of milk, this distinction seems an artificial one, the only well-marked separation being between those which coagulate and those which do not.

Fricke classes as Friedländer several organisms which ferment lactose and coagulate milk, which does not agree with the original description by Friedländer.

Potato.—Much emphasis has been laid on the growth of this group of organisms on potato, and indeed Fricke and others have gone so far as to attempt a definite classification on this basis. Clairmont does not, however, share his views, and the appearance of the cultures in this series leads to the belief that, while it is a very favorable medium for the growth of the organisms, there are no constant characteristics which enable us to say whether we are dealing with any special member of the group.

Several series of potato were inoculated, and the differences of growth of the same organism on successive samples were very

striking. In one series there was insufficient water with the potato, and gas formation was very rare, while where more water was present this was a conspicuous feature.

On the moist potato, on which the growth is probably most typical, there were in general two types of growth.

I. Profuse, moist, yellowish, growing somewhat darker with age; surface shiny; edges sharply defined; gas bubbles in the growth in varying amount; odor aromatic. This type includes Nos. 1-5, 7, 8, 10-19, Hopkins Nos. 1, 2, *B. pseudo-pneumonicus*, Howard, Wright, and Mallory. Under this head may also be included No. 9, *B. aërogenes*, Pfeiffer, Blumer, and *B. acidilactici*, which differed only in the variability of their gas formation.

II. Growth rather more thin and dry, often waxy in appearance; sometimes a few gas bubbles were seen, but usually there were none. This includes No. 6, Hopkins Nos. 3-6, Friedländer, Fasching, *B. capsulatus septicus*, *B. ozænæ* Abel.

B. rhinoscleromæ and *B. sputigenus crassus* gave a thin transparent watery growth spreading over the surface.

These appearances varied on different potatoes, but the average results are as above. To illustrate the variations possible on this medium, it is sufficient to note that, whereas Clairmont describes the growth of Wright and Mallory as thin and transparent, only to be seen by the shining appearance of the potato, it was found here to be the most active of all, with profuse gas formation. In his culture of *B. rhinoscleromæ* from Prague the same author obtained a somewhat raised, dirty growth, while my culture from the same source grew scarcely at all on this medium.

Discoloration of the potato also varied. In No. 2, Hopkins No. 3, *B. pseudo-pneumonicus*, and Blumer it was always marked after a few days, while it was never seen in *B. capsulatus septicus*, *B. ozænæ* Abel, or *B. rhinoscleromæ*. In all the others no rule could be made.

In spite of such discrepancies, Fricke contends that the growth on potato is sufficiently regular to admit of classification, and divides his organisms into two groups, as follows:

I. Yellow, profuse, sharply defined growth, of the consistency of thin salve; surface moist, irregular, glistening; growth soon

covering the surface. In this class he places Friedländer and Pfeiffer.

II. Profuse, moist, viscid growth, almost colorless, spreading rapidly; no discoloration; no gas formation. This includes *B. ozenæ* Abel.

In my series, Pfeiffer's bacillus and Friedländer always reacted differently from one another, but this difference from Fricke may lie in the fact, above stated, that his Friedländer acted on sugars in a manner similar to Pfeiffer, and was therefore probably not a Friedländer at all.

Bouillon.—Both dextrose-free bouillon, made up from different samples of meat, and ordinary bouillon, made up with Liebig's extract, were used with the organisms studied. The inoculated tubes were placed in racks in the incubator, and so arranged that they could be examined without disturbing them. By this means the statement of Clairmont was confirmed, in which he notes that there is practically always a pellicle of more or less delicacy, easily broken up and not re-formed. This pellicle varied markedly in amount and consistency with different samples of bouillon. In some cases the growth was much more marked than in others, and the amount of pellicle varied directly with the profusion of the growth, as might, indeed, be expected. Where the surface growth was marked, there was usually a white ring at the margin.

In all cases the medium was uniformly clouded, and a flocculent, but rather heavy, sediment was formed in varying amount. On shaking, this was readily diffused through the medium, and did not settle completely for some hours. The consistency of the bouillon was not changed, save that it was made slightly denser by the presence of the sediment, except in No. 9, and sometimes in *B. rhinoscleromæ*, in which the fluid showed marked viscosity.

INDOL REACTION.

As recommended by Theobald Smith, tests for indol were made in dextrose free bouillon, determined by control to be free from indol. The cultures were kept in the incubator for eight days, and tested by the method recommended by Lehmann and Neumann. To each tube $\frac{1}{2}$ volume of 10 per cent. sulphuric acid

was added, and the mixture heated to 80° C. It was then allowed to stand for a few minutes, in case the reaction should take place without further treatment. In any case, a drop of $\frac{1}{2}$ per cent. sodium nitrite was added, and one or two more if necessary. The reaction was noted, and further observations were made at the end of twenty-four hours.

In all but three cases—Hopkins No. 6, *B. pseudo-pneumonicus*, and *B. rhinoscleromæ*—a positive reaction was obtained, marked in Nos. 5–9, 18, 19. Hopkins Nos. 1, 3, 4, *B. sputigenus crassus*, *B. capsulatus septicus*, Pfeiffer, *B. ozænæ* Abel, *B. acidi lactici*, and Blumer, and slight in the rest.

In *B. acidi lactici*, Blumer, and No. 9 there was usually, though not always, a positive reaction without the addition of nitrite. These three organisms have been grouped together because of other similarities, and it is interesting that they should conform also in this. The reaction was not absolutely constant in any case, and Clairmont was unable to obtain it at all; so too much stress must not be laid on it.

FERMENTATION TESTS.

In this part of the work dextrose free bouillon was used as a basis. It was prepared according to the method of Theobald Smith, and titrated to + 1.5. To the media thus prepared 1 per cent. of the various carbohydrates tested was added, and the mixtures when placed in fermentation tubes and sterilized by the fractional method. All chemicals were chemically pure. The following were the carbohydrates used:

Monosaccharids	-	-	-	Dextrose and levulose
Disaccharids	-	-	-	Saccharose, lactose, maltose
Pentoses	-	-	-	Arabinose
Triatomic alcohol	-	-	-	Glycerin
Hexatomic alcohol	-	-	-	Mannite

In the first series of tests each sugar was taken up separately, and all organisms inoculated into the same batch of bouillon. Later, each organism was inoculated into all the sugars, made up with one batch of bouillon. This was done because well-marked differences occurred in different batches of bouillon.

The fermentation tubes were inoculated from recent agar cultures, and kept in the incubator at body temperature. They were examined daily as long as gas formation continued, and daily record was made of the amount of gas, the reaction of the open bulb, and the appearance of the media in the stem. When no gas had been formed for twenty-four hours, the equation $H:CO_2$ was estimated with 2 per cent. NaOH in the usual manner. In all cases where gas formation was weak, or where no gas was formed in one or more of the substances tested, or where the organisms had been a long while without passage through animals, inoculations were made in an attempt at standardization. Unfortunately, most of the cultures from Prague had apparently quite lost their pathogenicity, and, in spite of large and repeated doses, would no longer kill animals. All cases which failed to make gas in one or more substances were tested a number of times with different samples of bouillon to secure accuracy of results.

As arabinose is not fermented by yeasts, representatives of the different groups were tested in its solutions, and were all readily able to break it up with the formation of gas.

The appended Table I shows the result of the inoculations. As noted above, some of the Cleveland series were so similar that all are not given.

From the standpoint of gas formation the organisms studied fall into three groups:

- I. *Those which ferment all the carbohydrates used.*
- II. *Those which ferment all but one or two.*
- III. *Those which ferment none at all.*

By far the majority fall into the first group, and in the following summary all not mentioned under Groups II and III belong under Group I, and will not be specified. Under Group II we have the following subdivisions:

1. *No gas in lactose.*—None of these coagulate milk. This includes Friedländer, Fasching, *B. crassus sputigenus*, *B. ozenæ* Abel, Wright and Mallory, No. 6, Hopkins Nos. 3-6. In this division the first three and the last gave no gas in glycerin or in glycerin agar.

2. *No gas in saccharose.*—All these coagulate milk. This

TABLE I.

NAME OF CULTURE	AFTER TWO WEEKS			DEXTROSE			LACTOSE			SACCHAROSE			LEVULOSE			MALTOSE			ARABINOSE			MANNITE			GLYCERIN		
	Milk Alk. after Hours	Milk Acid. No	Milk Coag. in Coar.	Gas in (Closed) Arm	Reaction of Bulb	Cloud in Arm without Gas	Gas in (Closed) Arm	Reaction of Bulb	Cloud in Arm without Gas	Gas in (Closed) Arm	Reaction of Bulb	Cloud in Arm without Gas	Gas in (Closed) Arm	Reaction of Bulb	Cloud in Arm without Gas	Gas in (Closed) Arm	Reaction of Bulb	Cloud in Arm without Gas	Gas in (Closed) Arm	Reaction of Bulb	Cloud in Arm without Gas	Gas in (Closed) Arm	Reaction of Bulb	Cloud in Arm without Gas	Gas in (Closed) Arm	Reaction of Bulb	Cloud in Arm without Gas
Fasching, Kral.	48	45	Ac.	48
B. aerogenes Kral.	49	Ac.
B. cras. sput. Kral.	49	Ac.
B. pseudo-purum. Kral.	49	Ac.
B. Pfeiffer, Kral.	49	Ac.
B. ozaene Abel, Kral.	49	Ac.
B. rhinoscleromae Kral.	72	48	Alk.
B. acid lactici Kral.	72	48	Alk.
B. pneum. Friedl., Kral.	48	Alk.
B. m. c. Hopkins, 1.	48	Alk.
B. m. c. Hopkins, 2.	48	Alk.
B. m. c. Hopkins, 3.	48	Alk.
B. m. c. Hopkins, 4.	48	Alk.
B. m. c. Hopkins, 5.	48	Alk.
B. m. c. Hopkins, 6.	48	Alk.
B. m. c. Blumer.	48	Alk.
B. m. c. Howard.	48	Alk.
B. m. c. Wright and Mallory.	48	Alk.
B. m. c. Cleveland, 1, 13.	48	Alk.
B. m. c. Cleveland, 2.	48	Alk.
B. m. c. Cleveland, 3, 14-17.	48	Alk.
B. m. c. Cleveland, 4.	48	Alk.
B. m. c. Cleveland, 5, 18, 19.	48	Alk.
B. m. c. Cleveland, 6.	48	Alk.
B. m. c. Cleveland, 7.	48	Alk.
B. m. c. Cleveland, 8.	48	Alk.
B. m. c. Cleveland, 9.	48	Alk.
B. m. c. Cleveland, 10.	48	Alk.
B. m. c. Cleveland, 11.	48	Alk.
B. m. c. Cleveland, 12.	48	Alk.

NOTE.—In the above table the decimals under "Gas in Closed Arm" indicate the proportion of the arm in which bouillon was displaced by gas. Under the head "Reaction," "Ac." = acid, "Alk." = alkaline. For convenience, amphoteric reactions have been classed as alkaline. The figures under "Cloud in Arm without Gas" indicate the number of hours of the persistence of the cloud.

includes Blumer, *B. acidi lactici*, No. 9. The last gave no gas in glycerin.

Under Group III there are only two organisms, Hopkins No. 5 and *B. rhinoscleromæ*. *B. pseudo-pneumonicus*, when received from Král, fermented nothing, but after passage through animals became more active and fell under Group I.

The *relative amount of gas formed* was extremely variable in successive inoculations of the same organism. Some samples of meat seemed more favorable than others for the development, and induced the formation of a larger amount of gas. This continued true, though, as far as could be seen, the method of preparation was identical, and the only inconstant factor was the meat. In some cases the amount of gas formed by the same organism in successive samples of meat bouillon varied as much as between 10 per cent. and 80 per cent. of the closed arm or stem. More than this, successive inoculations into a series of sugars sometimes gave more gas in dextrose, less in lactose, and more in saccharose than in the former trial, or some other similar variation. For this reason statements as to the relative amounts of gas formed can be general only.

Strong, in his Group I (Friedländer, Wright, and Mallory, *B. ozenæ* Abel, *B. sputigenus crassus*, and probably *rhinoscleromæ*), finds the gas in saccharose about one-half the closed arm, in dextrose about one-third, and in lactose about one-third or less. This corresponds fairly well with our averages, except that in this group the gas in lactose is nil.

In his Group II (Pfeiffer, *B. aërogenes*, Kruse), Strong states that gas is made in all three sugars, in about equal amounts, perhaps least in dextrose. In the series under discussion *B. aërogenes* agrees fairly well, but Pfeiffer constantly shows twice as much gas in dextrose and saccharose as in lactose. The organisms obtained in Cleveland, which, with two exceptions, belong to this group, sometimes make as much as 80-90 per cent. of gas in dextrose, and only 20 per cent. in lactose and saccharose.

This variation does not interfere in any way with the more absolute classification based on the presence or absence of gas in these solutions.

In all of his cultures Strong obtained fermentation of saccharose, and considered this sugar as of least value, while in the present work three organisms can be sharply separated from the rest by this means.

Leaving aside for the present the most-used carbohydrates, the others may be dismissed quite briefly.

Levulose is of little value as a factor in differential diagnosis. Gas is formed up to about 40 per cent. of the closed arm, by all organisms which ferment dextrose. The equation $H:CO_2$ varies between $\frac{3}{1}$ and $\frac{1}{1}$.

Mannite may be dismissed with about the same statement. The gas usually fills about 50–80 per cent. of the closed arm; $H:CO_2 = \frac{4}{1}$ to $\frac{2}{1}$.

Maltose is also readily fermented, in a manner almost identical with mannite, all dextrose fermenting organisms making 50–80 per cent. of gas, with the equation $H:CO_2 = \frac{2}{1}$ to $\frac{1}{1}$.

Arabinose is similar in its reactions. It was not tried in all cases, but in several representatives of each class, and was found to be fermented by the dextrose fermenters, with formation of from 30 to 80 per cent. of gas in the closed arm, $H:CO_2$ being about $\frac{1}{1}$. The fact that it is not fermented by yeasts made it of interest in this line, but it seems to be of no more value than the three preceding.

Glycerin.—As a general thing, it is easily fermented, but when an organism begins to weaken in its fermentative powers, this seems to be the first to resist. In the present series, Friedländer, Fasching, *B. ozænæ* Abel and *B. sputigenus* crassus, which form no gas in lactose, and *B. pseudo-pneumonicus*, which do ferment lactose, form no gas in glycerin. It is noteworthy in this connection that Fricke mentions that he was unable to obtain gas in glycerin agar with his culture of Friedländer.

No. 6, the only one in the series which did not ferment lactose, had no effect on glycerin, and No. 9, which did not ferment saccharose, was similarly inactive. On the other hand, the Hopkins cultures, while unable to ferment lactose, fermented glycerin readily. This suggests to us that the lack of fermentation of glycerin may be a loss, more or less temporary, rather than a

definite characteristic. Of course, it may be said that the lack of lactose or saccharose fermentation may be of similar origin, but the fact observed in the class in bacteriology, that occasionally gas forming cultures of various groups suddenly refuse to make gas in glycerin, and the necessity of a good deal of further and cumbrous subdivision of the classes made, if this be accepted as a differentiation, strengthen my belief in the irregularity of the glycerin reaction. The amount of gas made varied in wide range, from a mere trace in some of the cultures received from Král, weak also in fermentation of other carbohydrates, to a full 100 per cent. of the closed arm in No. 11. $H:CO_2 = \frac{4}{1}$ to $\frac{3}{2}$.

The reaction of the open bulb, taken at intervals of twenty-four hours, in the inoculated tubes of the different sugars, was somewhat variable, as seen in the table. As noted by Clairmont, Fricke, and others, there is an alkali formation, which is either coincident with the acid formation, or follows rapidly upon it. After fermentation and acid production cease, this continues and may overneutralize the acid already formed. This alkali production varies a good deal, as may also be noted from the table, in which the reactions are taken after gas formation has ceased for twenty-four hours, or after six days in those cases where there was no gas formation at any time.

In the organisms which ferment dextrose and lactose with the formation of acid, there is usually enough acid formed to counteract the alkali in the open bulb, and the reaction remains the same as the original, unless the test is made some time after the cessation of gas formation, in which case the reaction of the bulb is always alkaline. In the other carbohydrates, the acid formation is often inadequate for the neutralization of the alkali, and so it is not infrequent to find alkali in the bulb while the production of gas is still active. In saccharose and in glycerin, even when the gas formation is so active as almost to empty the closed arm of fluid in twenty-four hours, this is often seen. Strong, Clairmont and others have estimated the acid formation, stating that $\frac{1}{30}$ c.c. of $\frac{N}{1}$ NaOH is required to neutralize 1 c.c. of a three-day culture. As a usual thing, the fermentation proceeds about that long, and in such cases my results and theirs agree fairly

well; but in some cases gas production continues for as long as ten days, and the continued formation of alkali in the open bulb leads to its diffusion through the fluid in the closed arm, with a consequent change in the reaction.

Taking up more specifically the organisms which failed to make gas in one or more sugars, it is important to determine whether they affect these sugars at all. Anaërobic growth cannot take place without the presence of some material which can be disassociated by the organism with the formation of oxygen, and in general the carbohydrates are the most suitable for this purpose. It is true that in the fermentation tube the condition is not one of true anaërobiosis, but this is approached to a fair degree, so that obligate aërobes will not develop in the closed arm. Growth in the closed arm is indicated by the presence of a cloud, and also by the formation of chemical products due to the growth of the organisms. The reaction of the closed arm is very difficult to obtain with accuracy, and small quantities of acid are almost certain to be obscured by the alkali formation noted in the bulb. Our observations are therefore confined to the *presence or absence of visible growth in the closed arm*.

In the organisms which did not form gas in *lactose*, No. 6, Hopkins No. 4, and Wright and Mallory in the stock cultures, gave a slight cloud for the first twenty-four hours, which disappeared later; but after these organisms were passed through guinea pigs, the growth was practically strictly aërobic. Hopkins No. 3, No. 6, Fasching, *B. sputigenus crassus*, and *B. ozænæ* Abel showed a slight cloud for the first twenty-four hours, and none thereafter. Friedländer from Král gave a cloud which persisted for forty-eight hours, and then faded away. Of the organisms which formed no gas in *saccharose*, all three gave a diffuse cloud in the closed arm in twenty-four hours, which was less in forty-eight hours and practically absent after that. In all these cases the reactions of the open bulb were either alkaline or amphoteric, and there was not enough acid in the closed arm to neutralize the alkali in a mixture of the two. These results indicate a *measure of growth in the closed arm*. The amount in no case was large, and no chemical determinations were made as to

the products of growth. It is possible, as noted before, that there might have been traces of dextrose in the bouillon, either from insufficient treatment with the colon bacillus, or from slight impurities in the sugars used. In the absence of accurate chemical tests, this cannot be absolutely determined. In any case, the constant characteristic of gas or no gas was not altered in any way.

In summation of the work on fermentation, it may be stated that in study of the organisms of this group the only ones of the carbohydrates which are of any value in differentiation are lactose and saccharose. Dextrose is valuable in the general classification, but not as a means of separation of the various members. Glycerin may also be of some value at times.

The only definite and constant division which seems justifiable from the study of the cultural characteristics is made by the separation of those organisms which ferment all carbohydrates from those which fail to ferment one or more. This agrees with Strong's ideas, but to his divisions must be added that one which does not ferment saccharose.

The rhinoscleromæ which came from Prague is apparently hopelessly degenerated, as it consistently refuses to make gas in anything. Strong had the same difficulty with his culture from the same source, but records that in another culture obtained elsewhere, and of more recent origin, the organism failed to make gas in lactose, while making it in dextrose and saccharose, and is probably to be considered as belonging in the same division as Friedländer.

In all the forms which were pathogenic, capsules were present in the animal body, and as a usual thing in milk, though in this medium they were sometimes much harder to demonstrate than in others. Many of the text-books say nothing about the capsules of *B. acidilactici*, but these were readily seen in milk. The form of the capsule and its reaction to stains did not seem to offer any satisfactory means of classification.

Passing on from the cultural characteristics to other means by which classification of this has been attempted, there remain pathogenicity, and its natural sequences, immunization and agglutination.

Pathogenicity.—This has been taken up by a number of observers, working with the usual laboratory animals, and quite a number of classifications have resulted. The most extensive, as well as the most recent, articles on this subject are those of Fricke, in 1895-96, and Clairmont in 1901-02, and their conclusions, based on their experiments, will be found in Table II.

TABLE II.
Pathogenicity.

NAME OF ORGANISM	CLAIRMONT			FRICKE—SUMMARY			PERKINS		
	M.	G. P.	R.	M.	G. P.	R.	M.	G. P.	R.
Friedländer.....	+	+	—	+	+	—	..	+	..
Pfeiffer	+	+	—	+	+	— + in large amount	..	+	..
Fasching.....	+	—	—	+	—	—	..	+	..
B. capsulatus septicus (P. proteus hominis)....	—	—	—	+	+	+	..	—	—
B. ozanæ Abel.....	+	+	—	+	—	—	..	—	—
B. rhinoscleromæ	—	?	—	+	+	—	..	—	—
B. crassus sputigenus (Kreibohm)	+	—	..	—	—
B. aërogenes	—	+	+	+	..
Wright and Mallory.....	+	+	+	..	+	..
Blumer	+	..

NOTE.—In the above table "M" = white mouse, "G. P." = guinea pig, and "R" = rabbit. The plus sign indicates the death of the animal, the minus sign its survival, and the combination of the two a variable pathogenicity, the more common result being uppermost. The "?" under B. rhinoscleromæ indicates that in the cases where death occurred cultures were negative. The mouse inoculations are subcutaneous, the guinea pig and rabbit intraperitoneal. In my own series of B. aërogenes the pathogenicity was variable, some killing rapidly, some slowly, and some not at all. The pathogenicity in my series was tested only as a means of standardizing the weaker organisms, and so is less complete than some of the others.

Fricke divides his organism into two groups, one of which is pathogenic for white mice, and little or not at all for guinea pigs, and another which is non-pathogenic for mice and kills guinea pigs readily. Clairmont divides his into three groups according

to their pathogenicity for mice, guinea pigs, and rabbits. The results, when tabulated, show that organisms from the same source may have diametrically opposite qualities, and Fricke gives no comparison of organisms described by others except by quotations from the original articles. Clairmont considers Friedländer, Fasching, *B. capsulatus septicus*, and Pfeiffer. He finds Fasching pathogenic for white mice only, Pfeiffer and Friedländer pathogenic for mice and rabbits, and *B. capsulatus septicus* not pathogenic. In my inoculations with the same organisms, obtained from the same source, Král's laboratory, *R. capsulatus septicus* was found to be non-pathogenic for guinea pigs, while Friedländer, Pfeiffer, and Fasching were readily and rapidly pathogenic. The single fact of the marked difference in the pathogenic properties of Fasching here and abroad, though the culture came from the same source, if accuracy in technic be admitted, is sufficient to throw out any classifications based on pathogenesis alone. Different breeds of the same animal may vary markedly in their reaction to bacteria, and it is also an accepted fact that organisms which are classed under the same head—*e. g.*, different strains of *Streptococcus pyogenes*, or of *B. coli*—may differ greatly from one another in their pathogenicity. For these reasons, classification on this basis seems unsatisfactory.

IMMUNITY AND AGGLUTINATION.

A number of attempts have been made to immunize animals against the organisms of this group, and to produce a protective serum. Among the earliest of these, Howard succeeded in immunizing guinea pigs against ordinarily fatal doses. Injection of their serum had, however, no protective effect.

Clairmont goes into the subject extensively, with discussion of previous work. The usual methods of immunization were used, and Pfeiffer's test as well, but though in many cases immunization was successful, the writer was led to the following conclusions:

„Die serodiagnostische Methode war damit als unbrauchbar erwiesen, und es musste auf jene schon oft benutzte Momente zurückgegriffen werden, um durch deren Erweiterung und genaue Feststellung unter möglichst gleichen Bedingungen die misslun-

gene Differenzierung nochmals zu versuchen. Wenn es sich früher als zweckmässiger erwiesen hatte, von bestimmten, aber nicht bindenden, vom Fundort genommenen Bezeichnungen der Stämme auszugehen, so musste jetzt jeder einzelne Stamm als solcher beschrieben werden, da ja die Bakterien in verschiedener Localität doch identisch sein konnten."

It seems as if the present knowledge of technic had been almost exhausted in the effort to separate the various members of the group one from another. The marked disagreement of different authors as to the appearance of the growths on agar, blood serum, gelatin, and potato, and in ordinary bouillon, show that there are no constant characteristics on these media except for the group as a whole. As just noted, pathogenicity and immunization are too variable to admit of making any hard and fast lines. The morphology, inasmuch as one of the main characteristics of the group is its pleomorphism, is of no great aid to us.

These close interrelations suggest the idea, brought out by several observers, that all these organisms may be descendants from one common stem. Some think this to be the Friedländer type, but on theoretical grounds it seems more probable that, if there is an original type, it is rather the "aërogenes" type, which ferments all carbohydrates, as it indicates a loss of power in the succeeding members of the group, rather than the acquisition of additional powers. Then, too, if Friedländer is the original type, the group which ferments lactose, but not saccharose, must have changed in a very roundabout way, or must bear no relation to the general group, to which, however, it is related closely both by all its cultural reactions and by its morphology.

After consideration of all these things, the only characteristic which has been found to be constant, and which is becoming daily of more importance in the classification of species, relates to the fermentation of chemically pure sugars, of which, as noted above, lactose and saccharose are most important as regards this group. Classification on this basis gives us three classes, easily distinguished from one another by the methods at hand in every well-equipped laboratory. Beginning with the largest and most active class, we have the following:

I. *All carbohydrates fermented with the formation of gas.*

II. *All carbohydrates, except lactose, fermented with the formation of gas.*

III. *All carbohydrates, except saccharose, fermented with the formation of gas.*

If this be admitted, it becomes necessary to find suitable names for these three divisions. Inasmuch as the best-known and earliest described of the organisms which fall into the first class is *B. lactis aërogenes*, its name should obtain. The name must conform to the rules of nomenclature, and accordingly that noted in Migula has been selected—*Bacterium aërogenes*, Migula, 1900. The prototype of the second class in Friedländer and the name under acceptance for that organism, under the rules above noted, is *Bacterium pneumonicum*, Migula, 1900. The third class has as its prototype *B. acidi lactici*, which is a satisfactory name for the organisms of this type.

B. acidi lactici has been classed by some with the colon group, but by most with *B. aërogenes*, usually with slight description. Hueppe, in the original description, described spores, but these have not been seen in any of the cultures by that name, received from various sources. Capsules, as noted above, were constant. The organism noted by Ford under the head of *Bacterium duodenale* answers exactly to the description of No. 9, and Blumer, as well as *Bact. acidi lactici*. Blumer's organism came from a case of septicemia, originating in acute enteritis; No. 9 came from a case of acute appendicitis; and Ford's *Bact. duodenale* came from the upper part of the intestine as a usual thing. The fermentation characteristics are very constant, inoculations of Blumer made in July, 1903, giving the same reactions as described by him in 1901.

The final classification is, then, as follows:

I. *Bacterium aërogenes*.—Under this head are included *Bacterium aërogenes*, *B. capsulatus septicus*, Pfeiffer and Howard, also Hopkins Nos. 1, 2, and all my series except No. 6.

II. *Bacterium pneumonicum*.—Under this head come Friedländer, Fasching, *B. sputigenus crassus*, *B. ozæne* Abel, Wright and Mallory, Hopkins Nos. 3, 4, 6, and No. 6 of my series. *B. rhinoscleronæ* probably comes within this division.

III. *Bacterium acidi lactici*.—Under this head come *B. acidi lactici*, Blumer, and No. 9 of my series.

SUMMARY AND CONCLUSIONS.

1. The name *Bacillus mucosus capsulatus* includes a large number of organisms described under various names, closely related morphologically and culturally, but differing in certain characteristics.

2. Animal inoculation and immunization and agglutination show results which are far too variable to admit of using them as means for classification.

3. Growths on the various ordinary laboratory media are subject to such great variations that they are also unavailable for purposes of classification.

4. Experiments in fermentation show constant results which may be used as a basis for the separation of the different members of the group, according to their ability to ferment lactose and saccharose.

5. The organisms of this group are widespread, and have been found in almost all pathological lesions which may be caused by bacteria.

6. Infections due to this group are exceedingly frequent in Cleveland, and appear to be due almost exclusively to the members of the *Bact. aërogenes* division.

7. These organisms may form gas from the body carbohydrates, either during life or immediately after death.

8. The portal of entry appears to be the nose and mouth, and secondarily the digestive tract.

It will be noted that, besides *B. rhinoscleromæ*, another organism, Hopkins 5, is described as forming no gas. In this case the organism was said by Dr. Harris, from whom it was obtained, to have formed gas at a previous time. In July, 1903, in spite of attempts to rejuvenate it, it was still unable to ferment the sugars. This, then, seems to be a case of powers lost, in a way similar to that seen in *B. rhinoscleromæ*; but another organism obtained through the courtesy of Dr. Blumer, and coming from a throat, was unable to grow in the closed arm at any time, being

apparently a strict aërobe, and was entirely non-pathogenic. Another similar organism, obtained from a similar source, came under my observation since the preparation of this paper, and the point has been raised whether organisms such as these, which possess the morphology, staining, and cultural reactions of the group, but have neither pathogenicity nor power to ferment carbohydrates, should be placed in a separate group.

Since, however, we have organisms which have once been able to ferment and now are no longer able to do it, it seems to me more natural to suppose that these non-fermenters belong to one of the regular groups, and have lost their chemical activities as well as their pathogenicity. It is quite possible that further work may show that such organisms should be placed in a section by themselves, but at present I will content myself with the above mention.

In conclusion, I desire to express my thanks to Dr. W. T. Howard, Jr., for the use of the material, and for his many and valuable suggestions in the course of the work. It is also a pleasure to express my acknowledgments to the trustees of the Rockefeller Institute for Research, for the financial assistance given during the course of the work.

REFERENCES.

- The literature on this subject has been so thoroughly worked out by Clairmont, and his list of references is so full, that I could have done little more than copy his work. It seemed better, therefore, to note here only such as were actually made use of, and those that Clairmont has omitted:
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 4. BLUMER AND LAIRD. *Bull. Johns Hop. Hosp.*, 1901, 12, p. 45.
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 6. ESCHERICH. *Darmbakterien des Säuglings*. Stuttgart, 1888.
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11. PERKINS. "Laboratory Epidemic Due to *B. muc. caps.*" *Jour. Exp. Med.*, 1901, 6, p. 389.
12. SCHEFFER. *Archiv f. Hyg.*, 1897, 30, p. 291.
13. WRIGHT AND MALLORY. *Ztschr. f. Hyg.*, 1895, 20, p. 220.

NOTE.—This article was prepared for publication July 1, 1902, but has been delayed until now in its appearance. The only article which has appeared since is that of Sachs, *Centralblatt für Bakteriologie*, Vol. XXXIII, Abt. I, p. 657. His work, as he himself admits, adds nothing new to the subject. He found an organism of this type in a case of pyonephrosis, at operation, and made a comparative study. The bacillus coagulated milk slowly, and often made gas in the milk culture. There is no record of the growth or chemical activities in lactose, in glucose, or in saccharose bouillon, but the coagulation and gas formation in milk place it in Class I or in Class III. The absence of records in regard to saccharose makes it impossible to say to which it belongs, but the source and the relative frequency of occurrence of the two make it probable that it should be placed as a member of Class I.

AGGLUTINATION IN THE GROUP OF FLUORESCENT BACTERIA.*

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VERY little has been done as yet in testing agglutination affinities among large groups of bacteria, although to some writers it has seemed probable that this method would throw light upon the true biological relationships of closely allied organisms. A few groups, however, have been closely examined with a view to the differential value of reciprocal agglutination.

A considerable number of cultures of *B. coli* isolated from the human intestinal tract were studied by Rothberger¹ and Radziewsky.² They draw from their work the conclusion that, as regards agglutination, a uniformity among the different cultures of *B. coli* tested does not exist.

Durham³ carried out an extended series of tests in an attempt to differentiate various organisms of the coli-typhoid group. He found that certain cultures of the *B. enteritidis* type which were to all appearance culturally alike showed no real similarity in their agglutinative reactions. It was also true that members of the *B. coli* group showed variability in their reactions even to very strong serum. On the other hand, cultures of "*B. coli communis verus*" and of "*B. coli communior*" gave positive agglutination tests with the same serum, although they were culturally different. He concludes, therefore, that "it is clear that the clumping reaction is of little value for differentiating and classifying these bacilli in a satisfactory manner" (p. 366).

Theobald Smith,⁴ in a valuable series of experiments, approaches the subject of bacterial agglutination as one phase of the relation between host and parasite. Cultures of the hog-cholera group isolated from man, the pig, the guinea pig, and the spermophile were tested as to "agglutination relationships" one to another and to related organisms such as *B. typhosus*, *B. coli*, and *B.*

* Received for publication February 1, 1904.

icteroides. The results of the tests led him to draw the conclusion that "bacteria isolated from well-defined types of disease, especially those known to invade the blood regularly, are clumped in nearly the same dilution." *B. icteroides* and one of the hog-cholera cultures, however, though differing culturally and in virulence, are clumped in the same dilution by one another's serum. Agglutination tests were made upon eight different cultures of *B. coli*, seven of which were isolated from the pig and one from the fowl; five of these were used in immunizing rabbits so as to determine the relation between the cultures. Of these eight cultures, four failed to ferment saccharose, and were hence considered by the author to have approached toward a "more pronounced parasitism." The other four he considered to belong to the true colon group. As a result of the tests, he found that, with one exception, the agglutination relationship runs nearly parallel with the biological characters and the specific parasitism. Finally, he concludes that "close agglutinative affinities may be predicted from close biological and pathogenic relationships."

Not many non-pathogenic bacteria have been tested with reference to their agglutinative reaction, but Kneass and Sailer⁵ have recently conducted some experiments with *B. subtilis*. In this work rabbits were treated by the subcutaneous injection of large numbers of the living bacteria. A high degree of agglutinative power was obtained, serum agglutinating in a dilution of 1:5,000 being secured in eighteen days. In testing the agglutination, the microscopic method with filtered broth cultures was employed. Complete agglutination of *B. subtilis* was obtained in two hours with a serum dilution of 1:2,500, and slight agglutination with a dilution of 1:5,000, but no reaction with a dilution of 1:10,000. The tests were made simply with the specific organism used in immunizing the rabbit.

AGGLUTINATION OF THE FLUORESCENT BACTERIA.

The writer has had the opportunity of studying the agglutinative reactions of forty-six different cultures belonging to the group of fluorescent bacteria, eighteen of these being non-liquefying and twenty-eight liquefying forms. These organisms had

all been isolated from river water. Dr. Jordan,⁶ in his paper on "The Kinds of Bacteria Found in River Water," has described the methods employed in isolating and studying the cultures I have employed. All of these fluorescent bacteria, with a very few exceptions, were isolated from forty-eight-hour-old gelatin plates, then "rejuvenated" in broth, 0.5 acid, for three days at 20° C., and finally plated in gelatin, from which agar cultures were made for the further examination of the organisms. Three of the organisms (1040 *y*, 1082 *w*, 1082 *x*) were isolated from the gelatin plate after passing through the dextrose broth fermentation-tube, and two others (1076 *l*, 1080 *m*) were isolated from litmus lactose agar plates after passing through carbol broth. These five forms, however, were "rejuvenated" and treated in the same manner as the other organisms of the group.

An examination of Table III accompanying Dr. Jordan's paper will show the main features of the morphology and biology of the fluorescent group. The liquefying type was easily distinguished from the non-liquefying by growth both in gelatin and milk, since all the forms that liquefied gelatin curdled milk with the production of acid, and then peptonized the casein, while the non-liquefying forms produced a strongly alkaline reaction in milk. Aside from these characteristic changes produced in milk and gelatin, the two groups were very similar, and the individual members of each group were practically identical morphologically and biologically.

These bacteria were all isolated and studied in the fall of 1901, while the present study of their agglutination was begun in the spring of 1903. The cultures originally isolated had been kept in stock in the laboratory under uniform conditions. When first examined by the writer, it was found that the power of production of fluorescent pigment had been lost by twenty-five cultures out of the forty-six. The cultural characteristics in gelatin and milk were studied carefully by the writer in the course of the work on agglutination, and found to agree in the main with those exhibited when first studied in 1901, except that curdling of the milk by the liquefying group did not occur; there was in 1903 simply a slight acidification, and this was at once followed

by rapid peptonization of the casein. Even a second rejuvenation of the liquefying cultures failed to cause initial curdling of the milk, such as occurred when the organisms were first isolated and rejuvenated eighteen months previously. There still remained, however, the same sharp line of demarkation between the liquefying and non-liquefying groups in respect to behavior in milk and gelatin; the liquefying forms appeared simply to have lost some of their power to produce acid.

METHODS.

The serum used in the agglutination tests was obtained from rabbits injected with specific organisms. Four rabbits were inoculated; two with culture 238 L₁, one with culture 1119 A, and one with culture 1080 M.

The inoculations were carried out in the following way: A suspension in physiological salt solution of a living forty-eight-hour-old slant agar growth of the organism was injected subcutaneously. Beginning with one-half of the scrapings of a forty-eight-hour-old slant agar growth, the amount injected was increased to the whole agar growth, then to two agar growths, and finally to four agar growths at a single injection. Rabbit No. 1, treated with culture 238 L₁, received twenty injections in the course of two months. Rabbit No. 2, also treated with 238 L₁, received eight injections in one month; in this case growth on two agar tubes was used in the first injection, and on the fifth inoculation four agar growths were used and continued. Rabbits Nos. 3 and 4, inoculated respectively with cultures 1119 A and 1080 M, were treated in the same manner as No. 1, but the inoculations were continued for three months in order to obtain agglutination in dilutions above 1:1,000.

The macroscopic method of testing agglutination was used. The cultures to be tested were grown in neutral sugar-free broth for from twenty-four to forty-eight hours at room temperature. The serum used was diluted with physiological salt solution. The serum was added to a measured quantity of the broth culture, so as to obtain the desired dilution of the serum in contact with the bodies of the bacilli, care being taken to obtain a uniform sus-

pension, so far as this could be determined by the eye. A control-tube was always made of each organism tested, and kept under the same conditions as the other tubes. All these tubes were then incubated for twenty-four hours, but were examined at intervals to note the extent of the clumping. Examinations were made at the end of one-half hour, one hour, two hours, three hours, four hours, and twenty-four hours.

Considerable difficulty was experienced at the outset in obtaining cultures of a uniform turbidity, because this group of bacteria tends to form surface films and a flocculent growth. The films, however, could generally be removed, and after some experience it was found practicable to use cultures at an age when they showed the least amount of flocculent growth. For the same reason four hours was found to be the usual limit at which the degree of agglutination could be best established, as the flocculent growth sometimes present after twenty-four hours obscured the reaction. The stronger sera and the lower dilutions of sera, however, sometimes gave a sharp reaction after twenty-four hours.

The serum lost very little of its agglutinating power after standing two weeks: no older serum was tried. Every time a serum was used after standing, and whenever fresh serum was used, tests were made with the homologous organism as well as with the other organisms, to determine whether the serum had lost any of its agglutinating power. Most of the serum used was not more than two or three days old.

The serum from rabbit No. 1, treated with *B fluorescens non-liquefaciens* 238 L₁, was first used in testing the agglutination of the other forty-five cultures of the fluorescent group. This culture was primarily selected because it was a thoroughly typical and vigorous form biologically, culturally, and in the production of fluorescence. Tables I and II show that of the eighteen other non-liquefying forms, ten gave positive agglutination; of the twenty-eight liquefying forms, fourteen gave a positive reaction, much variation, however, being shown in the degree of dilution of the serum by which they were clumped. Clumping occurred with 238 L₁ in dilution of its serum 1:10,000, while the limit of agglutination of most of the others was with serum diluted only

1:100. Five gave a positive agglutination with dilution 1:5,000, and five with dilution 1:1,000. The agglutination affinities between the liquefying and non-liquefying groups, and between the members within the groups, do not correspond with the biological characteristics.

It might perhaps be expected, first, that, since the organism used for inoculation belonged to the non-liquefying group, the other members of the non-liquefying group, being to all appearances biologically similar, would show practically the same agglutinative reaction; and, second, that there would be some distinction between the liquefying and non-liquefying groups; but in neither case can any such relation be traced.

In view of the results with immune serum 238 L₁, it was decided to inoculate three other rabbits; rabbit No. 2 with 238 L₁, since rabbit No. 1 had died in the interval; rabbit No. 3 with culture 1119 A, a member of the liquefying group of fluorescent bacteria and one agglutinated by immune serum 238 L₁ in dilution 1:5,000; and rabbit No. 4 with culture 1080 M, a member of the non-liquefying group, but not agglutinated by serum 238 L₁. It was found much more difficult to obtain a powerful serum with cultures 1119 A and 1080 M than it was with 238 L₁, it being necessary to extend the inoculations over a longer period, although a larger number of bacteria were inoculated each time. The writer was unable to raise the strength of the serum of rabbit No. 3, immunized against 1119 A, beyond the point of agglutination with serum diluted 1:4,000, and rabbit No. 4, immunized against 1080 M, beyond serum dilution 1:2,000.

Tests were made with the sera 1119 A and 1080 M on the forty-six fluorescent cultures just as in the case of serum 238 L₁. With serum 1119 A, eight of the eighteen non-liquefying forms gave a positive agglutination reaction, while this was true of only six of the twenty-eight liquefying forms, although 1119 A is a member of the liquefying group. With serum 1080 M, nine of the eighteen non-liquefying forms, and four of the twenty-eight liquefying forms, gave a positive reaction, 1080 M being a member of the non-liquefying group. As in the case of serum 238 L₁, the tests with sera 1119 A and 1080 M show no agglutinative

relationships between the liquefying and non-liquefying groups, or between the members within these groups.

Table III shows in comparison the agglutinations produced by the three sera. Serum 238 L₁ agglutinates a larger number and in higher dilution than either of the other two sera, probably because it is the most potent. Serum 1080 M, although somewhat weaker than 1119 A, agglutinates almost exactly the same number of cultures as serum 1119 A, and in about the same dilution. Mutual clumping reactions do not exist between these three cultures; for culture 238 L₁ is not agglutinated by either serum 1119 A or 1080 M; culture 1119 A is not agglutinated by serum 1080 M, but is by 238 L₁; culture 1080 M is not agglutinated by serum 238 L₁, but is by 1119 A. Culture 1119 A is agglutinated by serum 238 L₁ in dilution 1:5,000, while with its own less highly immunized serum it is agglutinated in dilution 1:4,000. The table shows further that not all the cultures agglutinated by serum 238 L₁ were clumped by serum 1119 A or 1080 M, nor were all the cultures agglutinated by serum 1119 A or 1080 M clumped in turn by serum 238 L₁. Nevertheless, it is true that, with a single exception, all the cultures agglutinated by serum 238 L₁, in dilution of 1:1,000, were also clumped by serum 1119 A or 1080 M in high dilution. It would appear that certain of the cultures were more sensitive to agglutination than others. Possibly if a higher immunity had been reached, more cultures would have been agglutinated. Although in general the strongest serum 238 L₁ clumped the cultures in higher dilutions than either serum 1119 A or 1080 M, there were a few exceptions. Culture 243 F₂ was agglutinated with serum 1119 A diluted 1:1,000 and not at all by serum 238 L₁; culture 1091 C was agglutinated by serum 1119 A diluted 1:500, while the stronger serum 238 L₁ agglutinated this culture only in dilution 1:100; culture 1021 D was agglutinated by serum 1080 M diluted 1:1,000, and by serum 238 L₁ diluted 1:100.

Table IV gives the varying degrees of agglutination shown by the three specific organisms used in these experiments. Complete agglutination, or that in which the clumped bacteria are completely precipitated, occurs at a lower dilution than the point of

just visible agglutination, or that in which the slightly clumped bacteria remain still suspended in the fluid.

To determine whether normal rabbit serum contained agglutinins sufficient to clump the fluorescent group of bacteria, tests were made with the serum of three different rabbits upon cultures 1119 A and 238 L₁ with negative results in every case, although low dilutions, 1:10 and 1:50, of the serum were used.

At the time of these agglutination experiments, there were in stock in the laboratory seven *Pyocyaneus* cultures from various sources, and five cultures of the fluorescent group which had been isolated from the human stomach. Agglutination tests were made with these organisms, using the three immune sera as in the other experiments, and showed that only one *Pyocyaneus* culture was clumped, and that only slightly, by serum 238 L₁ in dilution 1:100. In view of the small number of cultures tested from the *Pyocyaneus* group, no definite conclusion is justified. Of the five fluorescent cultures isolated from the human stomach, two of the three non-liquefying forms were slightly clumped by the serum of 238 L₁ (1:100).

I wish to express my thanks to Dr. Jordan, under whose direction this work was undertaken and carried on, for his valuable suggestions, and especially for his guidance and encouragement toward independent work.

CONCLUSIONS.

1. In the case of the group of fluorescent bacteria there appears to be no definite relation between the biological characteristics and the agglutinative reaction.

2. Certain of the cultures are more sensitive to agglutination than others, for, with one exception, the cultures agglutinated by serum 238 L₁ diluted 1:1,000 + were agglutinated by sera 1119 A and 1080 M in high dilution.

3. The strongest serum agglutinates the largest number of cultures and in the highest dilution.

4. These non-pathogenic organisms are less sensitive to the agglutination test than the parasitic and semi-parasitic bacteria of the colon-typhoid group.

NAME	DILUTION 1:40					DILUTION 1:100				DILUTION 1:1,000				DILUTION 1:5,000				DILUTION 1:10,000							
	½ hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.	½ hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.	½ hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.	½ hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.	½ hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.
288 L ₂	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+
1119 A	-	+	+	+	+	-	+	+	+	+	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+
185 Z.	-	+	+	+	+	-	+	+	+	+	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+
1022 B.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2081 D ₂ .	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1021 D II	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1002 A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1040 Y	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2049 A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

TABLE II—Continued.

NAME	DILUTION 1:40					DILUTION 1:100					DILUTION 1:1,000					DILUTION 1:5,000					DILUTION 1:10,000				
	$\frac{1}{2}$ hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.	$\frac{1}{2}$ hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.	$\frac{1}{2}$ hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.	$\frac{1}{2}$ hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.	$\frac{1}{2}$ hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.
1091 C	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1043 D.	+	+	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1069 A	-	-	-	+	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2069 A.	-	-	-	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1070 A.	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
82 B.	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1092 B.	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
					24 hrs					24 hrs					24 hrs										
1043 B.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2075 D.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1015 A.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2073 E.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1076 L.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2073 E.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2069 D.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1079 A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1082 X.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1080 M.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1044 W.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1059 B.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

TABLE III.

Comparison of the Agglutination of the Fluorescent Group of Bacteria Produced by Sera from Three Rabbits Immunized Respectively to 238 L₁, 119 A, and 1080 M.

FLUORESCENS- NON- LIQUEFACTENS	LIMIT OF AGGLUTINATION WITH			FLUORESCENS- LIQUEFACTENS	LIMIT OF AGGLUTINATION WITH		
	Serum 238 L ₁	Serum 119 A	Serum 1080 M		Serum 238 L ₁	Serum 119 A	Serum 1080 M
238 L ₁	10,000	(No aggl.)	(No aggl.)	238 L ₂	5,000	(No aggl.)	(No aggl.)
1070 D	5,000 ±	(No aggl.)	1,000	1119 A	5,000	4,000	(No aggl.)
2121 A ₁	1,000 ±	1,000	1,000	185 Z	5,000 ±	1,000	1,000
2121 A ₂	1,000 ±	1,000	1,000	1022 B	5,000 ±	1,000	1,000
198 L	1,000	1,000	(No aggl.)	2081 D ₂	1,000 ±	(No aggl.)	(No aggl.)
225 B ₂	1,000 ±	1,000 ±	1,000	1021 D fl	100	(No aggl.)	1,000
225 B ₁	100	(No aggl.)	(No aggl.)	1092 A	100	(No aggl.)	(No aggl.)
173 B	100	(No aggl.)	(No aggl.)	1040 Y	100	100 ±	(No aggl.)
116 G	100	(No aggl.)	(No aggl.)	2049 A	100 ±	(No aggl.)	(No aggl.)
1082 W	100 ±	100 ±	100 ±	1091 C	100 ±	500	(No aggl.)
243 F ₂	(No aggl.)	1,000	100	1043 D	100 ±	(No aggl.)	100 ±
1080 M	(No aggl.)	500 ±	2,000	1069 A	100 ±	100 ±	(No aggl.)
114 A	(No aggl.)	40	40	2099 A	100 ±	(No aggl.)	(No aggl.)
1082 X	(No aggl.)	(No aggl.)	100	1070 A	100 ±	(No aggl.)	(No aggl.)

TABLE IV.

Degrees of Agglutination of 238 L₁, 1119 A, and 1080 M by Their
Respective Sera.

Dilution hrs.		238 L ₁		1119 A		1080 M	
1: 40	$\frac{1}{2}$	+++	Complete	-		-	
	1	+++	Complete	++	Strong	+	Visible
	2	+++	Complete	+++	Complete	+++	Complete
	3	+++	Complete	+++	Complete	+++	Complete
	4	+++	Complete	+++	Complete	+++	Complete
1: 100	$\frac{1}{2}$	+	Visible	-		-	
	1	+	Visible	+	Visible	+	Visible
	2	+++	Complete	+++	Complete	+++	Complete
	3	+++	Complete	+++	Complete	+++	Complete
	4	+++	Complete	+++	Complete	+++	Complete
1: 200	$\frac{1}{2}$	+-	Slight	-		-	
	1	+	Visible	+	Visible	+	Visible
	2	+++	Complete	+++	Complete	+++	Complete
	3	+++	Complete	+++	Complete	+++	Complete
	4	+++	Complete	+++	Complete	+++	Complete
1: 400	$\frac{1}{2}$	-		-		-	
	1	+	Visible	-		-	
	2	+	Visible	-		-	
	3	+++	Complete	+	Visible	+-	Slight
	4	+++	Complete	+	Visible	+	Visible
1: 600	$\frac{1}{2}$	-		-		-	
	1	-		-		-	
	2	+	Visible	-		-	
	3	++	Strong	-		+-	Slight
	4	++	Strong	+	Visible	+	Visible
1: 800	$\frac{1}{2}$	-		-		-	
	1	-		-		-	
	2	+-	Slight	-		-	
	3	+	Visible	-		+-	Slight
	4	+	Visible	+	Visible	+	Visible
1: 1,000	$\frac{1}{2}$	-		-		-	
	1	-		-		-	
	2	+	Visible	-		-	
	3	+	Visible	+	Visible	+	Visible
	4	+	Visible	++	Strong	+	Visible
1: 2,000	$\frac{1}{2}$	-		-		-	
	1	-		-		-	
	2	-		-		-	
	3	-		-		+-	Slight
	4	+	Visible	+	Visible	+	Visible
1: 2,000	$\frac{1}{2}$	-		-		-	
	1	-		-		-	
	2	-		-		-	
	3	-		-		-	
	4	+	Visible	+	Visible	+	Visible
1: 2,000	$\frac{1}{2}$	-		-		-	
	1	-		-		-	
	2	-		-		-	
	3	-		-		-	
	4	+	Visible	+	Visible	+	Visible
1: 2,000	$\frac{1}{2}$	-		-		-	
	1	-		-		-	
	2	-		-		-	
	3	-		-		-	
	4	+	Visible	+	Visible	+	Visible

TABLE IV.—*Continued.*

Dilution	hrs.	238 L ₁		1119 A		1080 M	
1:4,000	$\frac{1}{2}$	—		—		—	
	1	—		—		—	
	2	—		—		—	
	3	+	Visible	—		—	
	4	+	Visible	+	Visible	—	
	24	+	Visible	+	Visible	—	
1:5,000	$\frac{1}{2}$	—		—		—	
	1	—		—		—	
	2	—		—		—	
	3	—		—		—	
	4	+-	Slight	—		—	
	24	+	Visible	—		—	

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STUDIES IN PNEUMONIA AND PNEUMOCOCCUS INFECTIONS.*

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INTRODUCTION.

IN this work, which was undertaken at the suggestion of Dr. Hektoen, an attempt has been made to establish more accurately (1) the frequency and time of pneumococcus invasion of the blood in pneumonia, and its relation to the leucocytosis, and particularly to the crisis, as well as the diagnostic and prognostic value of blood cultures in this disease; (2) to study the aggluti-

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nating, bactericidal, and other actions of pneumonic and other blood serum. In the course of the work attention became directed also to certain phases of the biology of the pneumococcus and of pneumococcus infection of the rabbit.

I. PNEUMOCOCCEMIA IN PNEUMONIA.

REVIEW OF THE LITERATURE.

The work of recent investigators shows remarkable differences in the frequency with which positive results have been obtained in blood cultures during life in lobar pneumonia.

The Klemperer brothers never found the pneumococcus in the blood, notwithstanding the fact that numerous cases were examined. Belfonti in many cases obtained only six positive results. Pässler found the *Streptococcus longus* and the bacillus of Friedländer once each in six fatal cases, while in thirty-eight cases that recovered the pneumococcus was found in the blood only once. Libman in 1894 demonstrated pneumococci in the blood during life in six of sixteen cases. In two of these they were found only in smears, the cultures remaining sterile. Three of the positive cases ended fatally, while only three of the twelve negative cases died. He used 5 c.c. of blood for agar plates. Kühnau, by the same method, but using twice the amount of blood, obtained only one positive result in nine cases. This case ended fatally. Two of the eight negative cases died. Kohn, also by the same method, grew pneumococci in nine out of a total of thirty-two cases. Seven of the positive cases died, while only two of the negative cases terminated fatally. Sereni in twenty-three cases found diplococci three times. Sello examined forty eight cases, with twelve positive findings. Ten of these ended fatally; twenty-seven of the negative cases recovered. Pieraccini, in 1899, found the pneumococcus in the blood in eleven out of twenty-eight cases of pneumonia. In 1899 White made cultures from nineteen cases, ten of which were fatal. Diplococci were obtained in but three cases: all of these ended fatally. He used 5 c.c. of blood in agar and bouillon tubes. Banti in 1890 found pneumococci in cultures in eighteen of twenty-nine cases. Cole in 1902 reports nine positive findings in thirty cases. All of the positive and four of the negative cases ended fatally. The earliest time at which cultures were obtained was three days before death. In four negative fatal cases cultures were made a number of times. Bouillon and litmus milk were principally used. The author lays considerable stress upon the importance of diluting the blood well, to "overcome its bactericidal action," and believes that the finding of the organism in cultures of the blood is of bad omen. Nazari found cocci in the blood during life in four of seven cases. Baduel recovered the pneumococcus from the blood in fifty-five of fifty-seven cases, in some cases as late as twenty-five days after the crisis. In 1900 Silverstrini and Sertoli report fifteen positive results in sixteen cases. They think that the number of pneumococci frequently, but not always, bears a direct relation to the severity of the infection. Berghini found them in every one of eight cases, and believes them to be

constant in the blood of pneumonics regardless of the gravity of the case. Casati as early as 1893 obtained positive results in twenty five consecutive cases by means of animal inoculations. Positive results were obtained as early as the second day. Fraenkel himself found the pneumococcus only in 20 per cent. of a large series examined, using the agar-plate method, but later he found them constantly by using bouillon as the culture medium. He shares the opinion of Prochaska that the organisms are present in every case, but that this does not necessarily mean a fatal prognosis. Prochaska in 1901 reports in two articles his results of blood cultures in fifty cases of pneumonia. In every case the diplococcus was found. Only one culture was made from each patient. He used bouillon and agar for his culture media, the former giving the more constant results. He lays no particular stress upon the degree of the dilution of the blood. Of the fifty cases, only twelve ended fatally. Positive results were obtained as early as the second day, and in one case as late as three days after the crisis. He believes his good results were due to the use of large quantities of blood (10 c.c.), in bouillon, and to great care in the examination of the cultures.

MATERIAL AND TECHNIC.

The cases examined were taken at random in the wards of the Cook County and Presbyterian Hospitals, and I am gratefully indebted to the attending physicians and the resident staffs for the interest taken and the many kindnesses shown.

After careful disinfection of the skin about the elbow by the use of green soap, 5 per cent. carbolic acid, and alcohol, the blood, 7 c.c. in the early and 10 to 13 c.c. in the later cases, was drawn from either the median basilic or cephalic vein. With great care to avoid contamination, so far as that is possible, 5-7 c.c. of blood was used for cultural purposes, while the rest was transferred to a 50 c.c. graduate, slanted until the blood coagulated, and then put into the ice-chest upright to permit the clear serum to drain off, which was then used for agglutination and other tests. In a small proportion of cases the white staphylococcus was found, presumably due to air infection, or perchance it was carried from the skin by the point of the needle. It is well to pass the end of the needle through a flame after withdrawing it so as to destroy all bacteria that may have been picked up from the skin, and that might otherwise be washed into the media with the blood while inoculating the flasks.

Bouillon (plain and glucose, 1 per cent.; acidity, 1 per cent.) and litmus milk mostly were used for the original cultures, the former giving the more constantly positive results, and of the two kinds of bouillon one proved about as good as the other. Glycerin bouillon (5 per cent.) was found to possess no special advantage over that of the others. In the early cases high dilutions were used (1:100 or 1:200), while later 1 part of blood to 50 or 75 parts of medium was employed. After incubation at 37.5° C. from eighteen hours to several days, smear preparations will show, usually, numerous diplococci. If the growth is at all abundant, a turbidity and brownish discoloration of the bouillon takes place, which is quite characteristic. The color reminds one of rusty sputum, and is due to the change in the hemoglobin of the blood.

Libman attributes this to the excessive acidity produced. The litmus milk is usually promptly acidified and coagulated. As pointed out by Cole, the capsule can usually be easily demonstrated in this medium. In all cases subcultures were made; the characteristic shape and staining reactions were noted in order to determine accurately the identity of the organism.

RESULTS.

Blood cultures.—In the total number of 145 cases studied, positive results were obtained in 132. In two cases (Nos. 35 and 24) the cultures remained sterile, but smear preparations directly from the blood showed the presence of undoubted pneumococci. A single blood culture was made in each of these cases on the eighth day of the disease. In the former of these there was a capillary leucocytosis of 42,000, while in the vein of the arm at the same time the blood showed a leucocytosis of only 30,000. In four of the negative cases (Nos. 10, 11, 15, and 38) cultures were made on the twelfth, thirteenth, fifteenth, and twenty-first days of the disease respectively, with normal temperatures in each instance. The blood was drawn mostly for other purposes and the cultures made incidentally. In two additional negative cases (Nos. 18 and 26) cultures were made on the second day of the disease. In case No. 104 the culture was made 36 hours after the crisis, with a negative result, and in No. 112 cultures were made on the day of the crisis, with a leucocyte count of 13,800. In only one of the negative cases (No. 55) is it difficult to explain the negative result. In this case three cultures were made, one on the day of crisis, one on the day following, and the third four days later, when there was an extension to the left lower lobe, after a previous massive consolidation of the entire right lung, in which resolution now appeared to be fairly well established. There was a leucocytosis of 30,000. Smears from the blood on the day of crisis, however, showed undoubted diplococci.

In most cases but one culture was made. However, in a number several cultures were made to determine any relationship that might exist between the crisis and pneumococcemia. The percentage of failures to obtain growths within thirty-six hours before crisis, and more especially on the day of crisis, seems to be higher than on the fourth or fifth day of the disease. Nor is the develop-

ment, as a rule, as luxurious. In four instances organisms grew after what appeared to be in each instance a typical crisis, but in only one of these was the growth at all abundant. Cultures were made from four to thirty-six hours after crisis in eight cases.

Otherwise positive results were obtained in all stages of the disease; in one instance, where pneumonia developed in a patient in the ward, the organism was obtained in pure culture twelve hours after the initial chill, before there were any definite physical findings. This case ran a typical course, with more delirium than usual. The pneumococcus was demonstrated in the blood in four other cases before a positive diagnosis could be made. Cultures were made six times on the second day, with three positive and three negative results; on the third day four times, with three positive findings. The fourth, fifth, and six days gave the highest percentage of positive results, except the period within the last twenty-four hours before death in the fatal cases.

The organism in three cases, especially, showed rather marked tendencies toward chain formation. Two of these showed distinct capsules, and resembled in every other way typical pneumococci; while the other, growing in still larger chains, with no capsule, but resembling very closely indeed the pneumococcus in its cultural characteristics, except that it only acidified milk, might be considered a variety of streptococcus; but from the fact that the serum of this patient (who, as the other two, passed through a typical attack) agglutinated pneumococci (as did also the others) of different strains, one might be equally justified in considering it a variety of Fraenkel's diplococcus. The tendency of the pneumococcus to form chains in some instances, especially when cultivated in milk, was well brought out in a number of observations. While making the routine subcultures on the various media, and also when purposely studying this point, it was noticed that very early in milk (twelve hours after inoculation) undoubted pneumococci would sometimes form long chains which resembled the chains of *Streptococcus longus* in every way, whereas later the typical encapsulated lanceolate diplococcus predominated upon the same medium, as it did from the beginning upon the other media. I have found the use of blood agar a good method for differen-

tiating pneumococci from streptococci (p. 308). It might be of interest to note that Dr. Weaver has found that pneumonic serum frequently possesses some, and at times very strong, agglutinating power over streptococci. Nine strains of streptococci were tested, obtained from various sources. Friedländer's bacillus was never observed in the blood cultures.

The mortality in this series of 40 per cent. shows that cases of rather marked severity have been studied, for the mortality of the total number of cases in the wards from which the cases were selected has been more nearly 35 per cent. Two cases of post-pneumonic gangrene showed pneumococci in the blood. The administration of antiseptics, such as creosote and guaiacol carbonate, do not seem to have any influence upon the presence of pneumococci in the blood.

Blood-smears.—By persistent search pneumococci were demonstrated in smears from the blood in forty-seven cases. In three of these the cultures remained sterile. Again, the organisms have been found repeatedly in the sera after being kept in the ice-chest for various lengths of time (in one instance as long as seventy days), and in three of these the original blood cultures also remained sterile. The positive results by this direct method of examination have been as numerous in the cases that recovered as in those that died. On the other hand, the negative findings are more numerous in the former. Various staining methods were tested, and it has been found that staining the film (after it has been fixed to the cover-glass in a corrosive sublimate-acetic acid solution) with Löffler's methylene blue is the most satisfactory. It may be contended that the diplococci found in the smears may have been contaminations; but the fact that chemically cleaned, sterilized cover-slips were used, carried to the bedside in sterile test-tubes, and replaced in the test-tube after the film had been made in a sterile manner, with forceps, then brought to the laboratory, placed in the fixing solution, and then treated with a carefully controlled staining solution, can leave little doubt that the diplococci found were present in the blood at the time it was drawn. The fact that the organisms were so numerous in some instances makes one raise the question

whether or not there might not have been a multiplication of germs in the blood upon the cover-slips. This possibility was excluded by fixing one film immediately before it was dry, and placing the other in the incubator for twenty-four hours. In every instance where pneumococci were found in the film which had been incubated they were also found in the film which was fixed immediately, and there appeared to be an equal number in both. In only three instances did I find what appeared to be diplococci within polymorphonuclear leucocytes. Other observers have found pneumococci in smears, Orthenberger in six fatal cases. Libman found them in cover-slips in two cases where the cultures remained sterile.

Diagnostic value of blood cultures.—In a number of cases in this series blood cultures have been of positive value in the diagnosis. In one case of severe sepsis in which typhoid was considered in the diagnosis the blood culture showed it to be a case of pneumococcemia probably due to a local inflammatory process in the nasal cavity. In six cases influenza or broncho-pneumonia was diagnosed, while the blood cultures contained pneumococci. In five cases pneumococci were demonstrated in the blood before any physical signs were present.

Prognostic value of blood cultures.—Authors differ remarkably in regard to the prognostic value of the demonstration of pneumococci in the blood. Fraenkel, Prochaska, Baduel, Berghini, Casati, and other recent workers attribute only small or no prognostic significance to pneumococcemia in pneumonia, because present in nearly all cases. On the other hand, Krauss and Sello, White, Pane, Cole, Pieraccini, and others consider invasion of the blood by pneumococci a bad sign. Why this great difference of opinion? In most instances conclusions are drawn from insufficient data; again, others seem to conclude that because they fail to grow the organism its absence is established—a most misleading conclusion. I believe the differences in the results are explainable wholly on the score of recent improvements in technic, to wit: the use of large quantities of blood in liquid media, especially bouillon, and of high concentration of blood instead of high dilutions to overcome the so-called bactericidal

action of the blood. All who have used the plate method have had positive results in only a small percentage of cases, while those who have used liquid media and animal inoculations have had almost uniformly positive results.

Eliminating the somewhat more uniformly positive results obtained during the last twenty-four hours of life, and the higher percentage of negative results upon the day of crisis in the cases with recovery, a careful analysis of the results earlier in the disease fails to show any difference in the number of times positive results were obtained in the fatal and non-fatal, and therefore less severe, cases. Hence we conclude with Fraenkel, Prochaska, Sittmann, and others that pneumococemia in pneumonia is of no special prognostic value.

II. PNEUMOCOCCEMIA IN RELATION TO THE LOCALIZATION OF LOBAR PNEUMONIA.

That pneumonia may be the secondary localization of a primary blood invasion does not seem altogether improbable, because the clinical picture of many cases of lobar pneumonia reminds one strongly of rapidly fatal pneumococemia with no special localization, and because in this series invasion of the blood stream seems to have been primary in point of time in five cases.

In the course of this work pneumonia resembling that in man resulted in two rabbits following intraperitoneal inoculation of pneumococci. The pneumococcus was isolated in pure culture from the blood in each instance. Washbourn has had similar results. Fraenkel, especially, mentions the development of pneumonia after injection with attenuated cultures, and Schultz has repeatedly produced a fibrinous pneumonia in rabbits by intravenous injections of attenuated pneumococci.

With the view of throwing more light upon this point, I undertook, with the aid of Dr. Rubin, a series of experiments upon six sets of rabbits (three in each set), of the same weight and inoculated with exactly equal doses of attenuated pneumococci, as follows:

Rabbit A in each set was inoculated intravenously, Rabbit B intratracheally, and Rabbit C also intratracheally, after having

been previously etherized by the hypodermic injection of 1 c.c. of ether per pound of weight. With the hope of attenuating the pneumococci sufficiently so that the rabbits would not die of a rapidly fatal bacteriemia, and thus afford time for the development, perchance, of a pneumonia, the washed-off pneumococci of one blood-agar slant were suspended in 85 per cent. salt solution and placed in the incubator for twenty to twenty-four hours, as advised by Auskoff and Schultz. This was found insufficient, for most of the animals died within a very short period. The animals inoculated intratracheally after having been etherized invariably died first, and the leucocytosis was not nearly so marked. The ones inoculated intratracheally usually succumbed before the one receiving intravenous injections, both dying usually of a rapidly fatal pneumococemia. This is interesting because it shows that the pneumococcus can penetrate into the circulation very rapidly from the respiratory tract, and may be considered a point in favor of the theory, advanced by Schultz and others, that the lung is the point of least resistance for the pneumococcus, and hence localizes there from a previous blood invasion.

Of the animals that survived long enough, there developed in two instances a pneumonia which resembled typical fibrinous pneumonia in man, and a bronchopneumonia in three others. In the former two it concerned intravenous, and in the rest, where the consolidation was not so sharply defined, intratracheal inoculations. In all of these there was a massive sero-fibrinous pleuritis, and in two a pericarditis. Pneumococci were isolated from the heart's blood in every instance. In a number of rabbits in this series, as well as in a large number of other rabbits which survived from twenty-four to forty-eight hours, or longer, the lungs frequently showed an active hyperemia in areas, which reminded one strongly of an early stage of red hepatization. To decide whether this condition was really an inflammatory process, and hence probably due to the pneumococcus, a number of specimens were placed in alcohol, sectioned, and stained for bacteria by Gram's method. In one of these I was able to demonstrate the presence of incapsulated diplococci within the alveoli, together with polymorphonuclear leucocytes, a large number of red blood

corpuseles, and other cells. This rabbit was inoculated intravenously, and died twenty-eight hours after inoculation; it was examined within a few hours after death.

We have thus shown (1) that the pneumococcus when injected into the trachea in lethal doses is rapidly absorbed by the blood; (2) that when injected intravenously it may enter the alveoli of the lung within the short time of twenty-four hours; (3) that a fairly typical lobar pneumonia may develop after intravenous and intraperitoneal inoculation; (4) we have confirmed the view, expressed by Fraenkel, that pneumonia in the rabbit is more prone to occur after inoculation with attenuated pneumococci. Hence it does not seem altogether unlikely that the lobar pneumonia in man may be the result of a primary hematogenous infection.

III. PNEUMOCOCEMIA IN RELATION TO THE LEUCOCYTOSIS AND THE CRISIS OF PNEUMONIA.

Accurate leucocyte counts were made simultaneously with the blood cultures in thirty cases. In four of these (see Table I), with a leucocytosis of 30,000 in two, and 13,800 and 12,340 respectively in the other two, the blood cultures were sterile. At first it would appear that this negative result might be due to a high leucocytosis, especially in the first two cases; yet from the fact that in the other two there was a low leucocytosis, and that in other cases positive results were obtained with equally high or even higher leucocyte counts, alike in fatal and non-fatal cases, it appears that there must be some other reason to explain these negative results of the blood cultures. Upon studying these cases more closely, it is found that in every instance except one the blood was obtained upon the day of crisis. Furthermore, in case No. 117, as the table will show, three cultures were made: one on the fifth day, with a negative result; one on the sixth day, with a positive finding; and again one on the seventh day, or the day of crisis, with a negative result. The leucocyte count remained practically the same (12,340). Again, in two of the fatal cases there was a marked reduction in leucocytes (1,600 and 1,040) shortly before death, whereas early in these cases there was a rather high leucocytosis (19,000 and 25,000). Clinically it has

been determined, especially by the researches of von Jaksch, that prognosis in pneumonia is more serious when leucocytes diminish in number. That this is true there can be but little doubt. This leads one to raise the question whether there exists any causal connection between the disappearance of the leucocytes and the appearance of pneumococci in the circulating blood.

A number of observers, more particularly Williamson, have attempted to show, from a rather small number of observations in man, and mostly by analogy from experimental pneumococcus infection in the rabbit, that this diminution of leucocytes and the

TABLE I.
Relation of Pneumococcemia to Leucocytosis and to the Termination of Pneumonia.

No.	Age	Day of Disease	Days before D'th or Crisis	Leucocytes	Blood Culture	Termination
19.....	27	7	0	6,000	+	Death
22.....	27	6	4	30,000	+	"
23.....	50	8	4	18,000	+	"
31.....	33	10	10	22,000	+	"
73.....	31	4	1	13,000	+	"
85.....	30	6	2	17,450	+	"
87.....	26	4	2	8,280	+	"
90.....	56	2	1	1,600	+	"
92.....	35	2	1	1,040	+	"
96.....	22	6	3	16,000	+	"
99.....	34	16	15	13,450	+	"
101.....	36	4	4	12,000	+	"
35.....	33	8	0	30,000	0	Recovery
49.....	31	3	3	22,900	+	"
55.....	19	8	3	30,000	0	"
77.....	28	7	4	36,550	+	"
88.....	15	6	2	17,250	+	"
89.....	23	8	Lysis	25,000	+	"
93.....	16	2	1	15,500	+	"
94.....	40	5	2	12,500	+	"
95.....	39	2	Lysis	10,640	+	"
98.....	35	8	2	5,473	+	"
100.....	40	7	2	17,350	+	"
102.....	40	2	2	15,000	+	"
103.....	36	5	2	14,250	+	"
108.....	30	10	2	22,000	+	"
109.....	40	7	2	23,600	+	"
112.....	35	7	0	13,800	0	"
116.....	40	5	3	19,450	+	"
		5	2	11,256	0	"
117.....	36	6	1	12,340	+	"
		7	0	12,000	0	"

total absence of leucocytosis in many fatal cases of croupous pneumonia are due to the "very special process" of the entrance of the pneumococci into the blood stream. Invasion of the blood by pneumococci occurs in nearly all fatal cases, and usually in large numbers, but this invasion is by no means to be regarded as an agonal event, because pneumococci have been found in the blood a number of times as early as the second or third day of the disease, and frequently as long as two, three, four, and a number of times six, days before death. Nor is blood invasion absent in the cases that recover, because it has not only been demonstrated in a large number of cases, by Prochaska, Fraenkel, Sittmann, Baduel, and in this series by myself, but the metastatic lesions in the most diverse organs observed every now and then in the course of even very mild cases of pneumonia show that the blood has contained the organism at least at some time during the infection. The practically constant pneumococcemia, even early in the disease in fatal cases, as well as in cases that ultimately get well, apparently independently of the number of leucocytes present, seems to indicate that pneumococcemia and leucocytosis in man are not necessarily so definitely associated, and that the diminution in leucocytes which does occur must be due to some other cause than the mere entrance of pneumococci into the blood stream. It may be more likely perhaps that the leucocytes are an index to the relative balance between the sum total of the infection, on the one hand, and the vital resisting agencies, on the other, and that hypoleucocytosis in pneumonia is an indication of failing resistance.

LEUCOCYTOSIS IN PNEUMOCOCCUS INFECTION OF THE RABBIT.

To test the conclusion drawn by Williamson that the diminution of the leucocytes in the pneumococcus infection of the rabbit is due to the penetration of the pneumococcus into the circulating blood, a series of experiments similar to his were instituted. After subcutaneous inoculation of lethal doses of highly virulent pneumococci, leucocyte counts simultaneously with blood cultures were made at frequent intervals, with these important additions: (1) Having previously shown that plain bouillon to which a small quantity of blood is added (7 loops per 5 c.c.) is a rather unfav-

avorable medium for the pneumococcus, and having repeatedly been able to obtain growths from old cultures of pneumococci upon blood agar, but not upon glycerin agar, I made parallel cultures in ascites bouillon, blood agar, plain bouillon, and glycerin agar, using the same number of loops as did Williamson for each inoculation. (2) Believing also that a knowledge of the actual number of viable pneumococci in a given definite quantity of the animal's blood, together with a simultaneous count of the leucocytes, might throw some light upon the rôle these cells play in the infection, a series of blood-agar plates (5 to 7 drops of sterile blood to 5 c.c. of plain or glycerin agar) were also used.

A study of Table II will show that by the use of more favorable media for the growth of the pneumococcus, and incidentally by the use of a somewhat larger quantity of blood, the organisms have been cultivated much earlier in many instances than in the experiments of Williamson, Radziewski, and others, and than I was able to do upon the media they employed. Since the pneumococci are usually numerous upon the plates at the time, we are able to grow them in plain bouillon and glycerin agar; and because of the fact that their demonstration in the blood, often in considerable numbers, by this method of examination is possible long before the acme of the leucocytosis has been reached (from three to twenty-four hours in the rabbits with an early death, and to as long as seventy-two hours in the ones that lived a number of days), it may be concluded (1) that the hypoleucocytosis which develops in the course of a fatal pneumococcus infection of the rabbit is not due to the mere entrance of the pneumococci into the circulating blood, but rather perhaps to the exhaustion of protective agencies; and (2) that invasion of the blood in the rabbit, as in pneumonia in man, is to be regarded as a part of the disease-process, and not as a "very special" or ominous event. The latter conclusion is still further borne out by the fact that two rabbits in this series recovered, in one instance with as many as 12,000 pneumococci per loop of blood (the loop used in all these experiments contains approximately 1/1,000 c.c.).

Here I would also note the recovery of a number of other rabbits (not in this series), which were inoculated subcutaneously,

after the presence of pneumococci had been demonstrated in the circulating blood.

That there exists a very close relation between the number of leucocytes and the number of pneumococci in the blood is evident, however, because in the fatal cases, up to a certain point, the rise in the number of leucocytes runs hand in hand with the increase in the number of pneumococci; again, after the leucocytosis has reached the acme, there is first a primary drop in both, just as if the disintegration of leucocytes causes a destruction of a number of the invading cocci (*vide* Table II, Nos. 1, 3, 5, 6, 8, and 10). This suggests that because of the exhaustion of the power on the part of the organism to produce new leucocytes, the pneumococci get the upper hand, and as the leucocytes diminish, the pneumococci increase in number. There is thus furnished by the blood-agar plate method a probable explanation of the observation, unaccounted for by Williamson, that at times his cultures remained sterile, while the earlier ones had shown pneumococci. This was associated in nearly every one of these instances with a rather sudden drop in the leucocytosis. Cultures upon blood agar would probably have shown pneumococci in the blood, though in diminished numbers, just as in my experiments.

In certain experiments in conjunction with Dr. Rubin it was found that alcoholized and etherized rabbits withstood pneumococcus infection much more poorly than normal rabbits; furthermore, that in the alcoholized and etherized rabbits the leucocytosis was not so marked, being at times wholly absent, while the number of pneumococci in the blood was greater than in the normal control rabbits.

These results seem to me to indicate that the leucocytes play an important rôle in the struggle against pneumococcus infection. This is in full accord with the fact that Kitasato and Wassermann, Buchner, and others have shown that *in vitro* the leucocytes have a definite bactericidal action (see Däubler, *loc. cit.*). The fall in the number of pneumococci shown by me to be coincident with the fall in the leucocytosis points directly to the liberation of pneumococcidal substances.

Filtrates of bouillon and serum cultures of pneumococci pro-

TABLE II.
Pneumococcemia and Leucocytosis in the Rabbit.

No.	Hours after Inoculation	Temperature (Rectum)	Leucocytes	BLOOD CULTURES						REMARKS
				Bouillon	Asites Bouillon	Gly. Agar	Blood Agar	Blood-Agar Plates		
								2 Loops	10 Loops	
1	$\frac{1}{2}$	101.8	11,340	0	0	0	+	0	10	Rabbit lived 1 mo. after inoc. At end of 3d week it developed pneumococ. arthritis of left knee joint; recovered. Death (finally) due to a massive pneumococ. pleuritis.
	4	102.4	12,840	0	+	0	+	15	0	
	12	103	15,250	0	+	0	+	25	144	
	24	100	18,840	+	+	0	0	30	165	
	48	102	8,440	0	+	0	+	7	30	
	54	104.8	3,520	+	+	0	+	15	100	
	66	101.8	10,520	0	+	0	+	7	40	
114	101.6	8,680	+	+	+	+	116,640	260,560		
2	$\frac{1}{2}$	102.2	10,800	0	+	0	+	0	0	Death 37 hrs. after inoc.
	2	102	7,400	0	+	+	+	6,500	48,200	
	16	102.8	4,800	+	+	+	+	34,200	+	
	28	104.4	1,320	+	+	+	+	22,680	544	
	32	101.4	1,160	+	+	+	+	+	+	
3	2	102.8	10,450	+	+	0	+	11,059	118,317	Recovery.
	16	103	5,120	+	+	0	0	3,110	13,895	
	28	105	12,380	+	+	0	+	50	25,344	
	40	102.2	11,380	0	0	+	+	0	120,086	
	48	104	10,300	0	0	0	0	0	0	
	63	104.7	0	0	0	0	100	0	
	87	104.6	12,360	0	0	0	0	0	0	
4	2	103	11,450	0	+	0	+	17,560	150,000	Death 25 hrs. after inoc.
	8	105	14,336	+	+	+	+	40,360	+	
	16	103.3	6,000	+	+	+	+	16,430	+	
	24	101	1,100	+	+	+	+	+	+	
5	$\frac{1}{2}$	101	10,900	0	0	0	0	Death 92 hrs. after inoc.
	4	102	12,460	0	+	0	0	
	12	103	18,360	+	+	0	+	100	1,204	
	24	103.5	24,000	+	+	+	+	340	675	
	48	105	5,700	+	+	+	+	60	128	
	72	105	3,700	+	+	+	+	+	+	
6	$\frac{1}{2}$	100	12,000	0	0	0	0	Recovery.
	4	101	13,430	0	0	0	0	0	0	
	12	102	14,280	0	+	0	0	0	0	
	24	103.5	16,000	0	0	0	0	0	0	
	48	103.6	25,400	+	*	0	0	0	1	
	72	103	27,000	0	0	0	0	75	
	92	104	25,300	0	0	0	0	0	0	
7	$\frac{1}{2}$	102	9,200	0	0	0	+	0	0	Death 85 hrs. after inoc.
	6	104.5	5,580	0	+	0	+	60	250	
	14	103.5	6,020	..	+	+	+	200	500	
	24	106.4	11,360	..	+	+	+	+	+	
	40	105	+	+	+	25	120	
	72	104	24,000	..	+	+	+	100,000	
	84	12,020	..	+	+	+	
8	$\frac{1}{2}$	9,600	0	0	0	+	0	0	Death 36 hrs. after inoc.
	3	103	21,560	+	+	+	+	188	1,778	
	6	103.8	13,120	0	0	0	+	84	166	
	12	104	12,450	+	+	+	+	45	237	
	24	104.5	11,100	0	+	+	+	100	1,442	
9	$\frac{1}{2}$	102.5	9,800	..	0	0	0	Death 49 hrs. after inoc.
	6	103.8	16,320	0	0	0	0	50	208	
	24	106.6	32,000	+	+	698	15,000	
	48	11,500	+	100,000	1,250,000	
10	$\frac{1}{2}$	102.4	9,845	0	+	0	0	0	0	Death 90 hrs. after inoc.
	6	104	14,000	0	+	0	+	0	0	
	24	105	38,700	+	+	+	+	8	74	
	48	103	14,120	0	0	0	+	2	14	
	72	98	12,600	+	+	+	+	1,234	5,678	
	85	99	8,870	0	+	0	+	6	34	

+ means very many.

duce in doses of 2-4 c.c. a slight rise of temperature and a considerable leucocytosis (18,000-28,000) in rabbits. Sterile bouillon and serum in the same quantities injected in the same way have no such action. This suggests that pneumococci may produce soluble toxins, and is in full accord with the observation that up to a certain point the increase in the number of leucocytes and in pneumococci go hand in hand in fatally infected rabbits.

IV. ACTION OF BLOOD AND SERUM ON PNEUMOCOCCI.

Media containing blood seems to be especially adapted for cultivating the pneumococcus. Washbourn advised the use of agar slants over which a few drops of rabbit's blood are allowed to flow as an excellent culture medium, and found that on it the pneumococcus lived much longer than is usual. Numerous parallel tests with different strains of pneumococci on rabbit's, human (both normal and pneumonic), dog's, and goat's blood-agar slants have been made in the course of this work. The efficiency of all these has been found the same. To determine further the constituent of the blood that especially favored the growth of the diplococcus, and thus to test the statement, made by Rymowitsch, that it was the hemoglobin, a hemoglobin infusion was made by laking washed corpuscles in distilled sterile water. The red fluid was floated over the surface of agar slants, protected from drying, and incubated for three days. Parallel tests were then made with the blood-agar slants and the hemoglobin medium; both media were found equally efficient, and both much more so than coagulated bovine serum, or glycerin agar, both as regards preserving vitality and securing abundance of growth.

In the course of certain experiments to determine the pneumocolytic effect of pneumococcus filtrates, it was shown that washed human and rabbit's corpuscles, when added to normal salt solution, increased materially the development and viability of pneumococci. The corpuscles at the same time were laked. By the use of blood-agar slants it has been found possible to preserve a moderate degree of virulence as long as 250 days, and in two instances a very high degree of virulence for 149 and 163 days. (In preparing blood agar plain agar preserves both vitality and

virulence better than glycerin agar.) The tubes were kept in the incubator at 37°C ., and drying was prevented by sealing with a sterile rubber cap the mouth of the tube after sterilizing in the flame. It is a well-known fact that the pneumococcus dies upon ordinary laboratory media in a very short time, and that even when transplanted frequently, and in this way kept viable, it loses its virulence very soon. The difference in the effect of ordinary glycerin agar and blood agar upon the pneumococcus is well brought out in the following experiments: A pneumococcus of standard virulence kept on blood agar was inoculated on the surfaces of three glycerin-agar and three blood-agar slants, which were placed in the incubator. At the end of twenty-four, forty-eight, and ninety-six hours, respectively, rabbits of the same weight were inoculated intraperitoneally. In each instance one rabbit was inoculated with pneumococci from the glycerin-agar and one with cocci from the blood-agar slant. The twenty-four-hour-old cultures produced death within twenty-four hours in both rabbits. After forty-eight and ninety-six hours of incubation, the two rabbits receiving the pneumococcus from the blood agar died promptly, while the other two, receiving the pneumococcus from the glycerin agar, survived. The forty-eight-hour-old culture produced some reaction, whereas the one incubated for ninety-six hours seemed not to affect the animal. Experiments like these lead one to believe that the conclusions drawn by numerous investigators upon the virulence or non-virulence of the pneumococcus, after having been grown on ordinary artificial media, are often erroneous and untrustworthy. Hence I would urge most emphatically, as Washbourn does, the use of blood agar, or other blood-containing media, in all investigations with the pneumococcus where, as is usual, a standard of virulence is indispensable for reliable results.

To determine further the influence of blood on pneumococci, parallel blood cultures were made in the following dilutions: 1:200, 1:100, and 1:8. Positive results were obtained in every instance of the 1:8 dilution, even when no pneumococci developed in the higher dilutions. This was constant with no exception. Now the chief difference between Prochaska's method, with uni-

formly positive results, and that of Cole was that the former used rather high concentrations of blood, and the latter rather high dilutions. The higher percentage of negative results in the early cases in this series, using higher dilutions than in the later cases, and the high total percentage of negative cases in Cole's series, I believe, can be attributed to this fact. Further, in a number of cases growths were obtained in pure pneumonic blood, when the cultures on bouillon and milk remained sterile. Case No. 116 showed this particularly well. The blood that was left after making the inoculations was defibrinated and placed in the ice-chest. Two weeks later a number of plates were made with blood agar, and there now developed in this medium an average of 350 colonies to the drop of blood. Whether there was any growth in the blood or not is difficult to say, yet it certainly shows that the blood drawn from a pneumonic patient possessed no bactericidal properties over the pneumococcus; and it also shows again that to conclude that pneumococci are absent in the blood because we fail to grow them on artificial media is incorrect. Further, by numerical determinations it has been shown that the pneumococcus multiplies rapidly when inoculated into fresh normal and pneumonic blood, so that blood in the medium, instead of having a bactericidal effect on the pneumococcus, distinctly favors its growth.

Has normal and pneumonic human serum any bactericidal action upon the pneumococcus? Behring found none; and the following reasons lead one to believe that such is the case: (1) Normal and pneumonic serum, especially when diluted with equal parts or more of bouillon, form a most excellent culture medium for the pneumococcus, growth being constantly more abundant than in bouillon only. The viability is increased if at the bottom of the tube blood corpuscles are present. (2) Living pneumococci in pneumonic serum survive for seventy-nine days when kept at 3° C.—a temperature to which the pneumococcus is otherwise very sensitive. (3) In Table III it is shown, by actual numerical determinations by the use of blood-agar plates, that normal human serum and blood and pneumonic serum appear to have no bactericidal power whatsoever over the pneumococcus.

The age of the sera in these tests varied up to twenty-four hours, and it was found that preserving the serum for twenty-four hours makes no difference. One-half c.c. of serum in each instance was inoculated with one loop of the water of condensation from blood-agar slants inoculated with the pneumococcus. After incubation (37° C.) for the different periods indicated in Table III, one loop was plated in blood agar and a careful count made of the number of colonies that developed. Blood-agar plates have the following advantages: (1) Owing to the small size and the

TABLE III.

Effect of Normal Human and Pneumonic Serum and Blood upon the Pneumococcus.

Serum	Pneumo- coccus	Immedi- ately	3 Hours	5 Hours	24 Hours	48 Hours
Normal serum W	R 49	100	180	250	15,860	18,200
Normal serum W	116	48	260	480	1,000	4,500
Normal serum D	× 239	12,180	7,385	13,350	2,205	4,690
Normal serum D	42	11,180	20,520	22,400	∞	4,340
Normal serum D	116	200	510	7,700	∞	7,200
Normal serum D	69	4,480	8,400	∞	∞
Normal serum S	1,386	1,004	1,824	3,684	40,000
Normal serum S	R 46b	848	1,368	1,824	10,456
Normal serum S	46	4,856	18,414	55,242	∞
Normal serum H	1,386	840	2,268	17,995	∞
Normal serum H	R 46b	912	1,386	7,824	∞
Normal serum H	47	16,800	29,127	∞	∞
Normal serum A	1,386	2,144	9,800	14,120	∞
Normal blood D	41	50	432	168	12,600
Normal blood S	R 46b	4,848	18,414	55,414	∞
Normal blood H	R 46b	6,912	32,352	∞	∞
Pneu. serum 116*	1,386	1,440	5,720	6,590	∞
Pneu. serum 116	41	108	5,720	10,450	∞
Pneu. serum 117†	R 49	1,360	1,540	1,470	∞	4,200
Pneu. serum 117	× 239	3,570	7,385	9,860	14,000	7,280
Pneu. serum 117	42	11,040	15,000	∞	11,340	5,390
Pneu. serum 117‡	R 49	140	280	∞	∞	4,270
Pneu. serum 117	116	66	33	78	280	13,700
Pneu. serum 117	× 239	12,600	16,280	21,160	5,775	6,930
Pneu. serum 117	69	7,280	7,560	∞	∞
Rabbit's blood 43	46b	6,200	14,722	∞	∞
Rabbit's serum 43	42	7,392	∞	∞
Controls milk	1,386	8,000	10,880	∞	∞
Controls milk	41	41	100	776	∞

* Blood was drawn on the day before crisis with a leucocytosis of 17,450.

† Blood was drawn on the day before crisis with a leucocytosis of 12,460.

‡ Blood was drawn on the day of crisis with a leucocytosis of 12,000.

∞ Stands for innumerable.

transparency of the colonies of the pneumococcus, their recognition on plain or glycerin-agar plates is made very difficult; this is removed to a large degree because upon this medium the colonies not only grow somewhat larger, but have a decided greenish color. (2) Careful controls with glycerin agar show that upon the blood medium there constantly develop a larger number of pneumococci, and thus by its use we obtain a more accurate knowledge of the number of viable cells present. (3) This medium affords the opportunity at the same time to study the hemolytic effect of the pneumococcus, and it has been found that highly virulent pneumococci (such as have been passed through a series of rabbits) possess a greater power of hemolysis than do those of a lower degree of virulence, and that the pneumococcus when grown in the serum, both natural and pneumonic, soon loses its hemolytic power. The diminution in the number of colonies which occurs after forty-eight hours' incubation is clearly due to exhaustion, and not to a bactericidal effect; for, where the number of micro-organisms was small in the beginning, and hence the development rather more slow, this diminution is not noticed, but a continued multiplication instead.

The pneumonic sera were carefully controlled in every instance with normal sera from different individuals. It has been found that, while there is no difference in the rapidity with which development of pneumococci occurs, nor with the rapidity of the death of the cocci later, there is this difference that there results early a marked turbidity, and later an abundant precipitate, together with agglutination, in the pneumonic serum, whereas the normal serum remains practically clear, containing, however, equally many cells. This marked turbidity and precipitation in pneumonic serum has led to the erroneous conclusion, by a number of investigators, that it was due to an enormously rapid multiplication of pneumococci.

V. AGGLUTINATION OF THE PNEUMOCOCCUS.

The pneumococcus when cultivated in the sera, as well as when injected into the body, of susceptible animals, appears in typical diplococcus forms, and occasionally in very short chains.

In the sera of less susceptible animals—*i. e.*, the dog, cat, or horse—Kruse and Pansini noted the tendency of the organism to grow in chains and bunches. Wadsworth, however, very recently states that he has been unable to confirm their findings. Metchnikoff first observed the tendency toward chain formation in the serum of immunized rabbits. Later Arkharow, Issaef, Mosney, Washbourn, and others noted that this tendency to the formation of chains and bunches in the serum of immunized animals was associated with what seemed to be definite evidences of degeneration of the organisms, and believed it to be closely related to the degree of immunization. Bensançon and Griffon considered the growth in clumps and chains in the sera of specially immunized animals so marked and characteristic that they described it as agglutination; they were the first observers to apply this reaction to human sera from croupous pneumonia and other pneumococcus infections. Their method consisted in inoculating 1–2 c.c. of undiluted serum with pneumococci, and observing the growth at the end of twenty-four hours. If the reaction was positive, the bottom of the test-tube would show a membrane or a gelatinous mass composed of clumps and chains of pneumococci. They were rarely able to obtain agglutination before the day of crisis, and they found the agglutinating substance to diminish rapidly thereafter.

Neufeld later studied the agglutinating action of the serum of immunized animals on pneumococcus-bouillon cultures. He used various dilutions up to 1:50, and was the first observer to note agglutination of pneumococci microscopically and macroscopically by the usual method. He emphasized the importance of using fresh serum, and found that agglutination took place in from one-fourth to one-half hour. Pneumococcus agglutination was studied by Huber, Daddi, and Pesci, and recently by Wadsworth, who has devised a new technic, which he believes is more reliable and delicate than the methods hitherto employed. In this work the reaction of blood serum from cases of croupous pneumonia or other pneumococcus infection was exclusively studied. For the controls, which were carefully made in every instance, normal human serum was used. Both the method of Bensançon and

Griffon, but using various dilutions of serum (as high as 1:50), and that of Neufeld were employed. And while the latter is no more delicate, it has the advantage that (1) results can be obtained in a shorter period; the process being complete in from one-half to several hours; (2) the microscopical changes in the process can be more easily watched; and (3) it eliminates practically the rapid growth of organisms which occasionally seems to occur, especially in the sera in which the complement has been destroyed, either by heat or age. It has, however, the decided disadvantage that it is often difficult to get a concentrated bouillon culture of pneumococci.

After twelve to twenty-four hours' incubation in the former method, and earlier in the latter, the liquid becomes perfectly clear, and the growth forms a membrane or a gelatinous mass at the bottom of the tube, which does not readily mix with the clear serum upon shaking, especially if the agglutination is marked. When the reaction is more feeble, and in the higher dilutions, a whitish, flocculent precipitate is thrown down. This readily mixes with the overlying liquid, which is usually more or less turbid. The sediment in each instance is early composed of swollen diplococci, single, in long chains or bunches, and late of a highly refractive, nonstaining, granular substance.

When equal quantities of agglutinating pneumonic serum and pneumococcus bouillon culture are placed in the hanging drop, the single cocci swell to twice and three times their normal size; the opposing ends become flattened, their outline indistinct, and the cells homogeneous. In the center of each coccus is seen a very small, round, highly refractive spot. Long chains and clumps are often seen. Ultimately they lose their affinity for the ordinary stains, as Neufeld has shown. These changes were further studied by the use of the capsular stains recommended by Welch, Hiss, and Boni. Frequently empty capsules and what appeared as degenerated fragments of cells in capsules, in clumps or single, were seen. Capsules of enormous size are often demonstrable early in the process. This entire picture gives the impression of a rapid lysis or degeneration. In short, the changes observed appear to be identical with those described, first by

Radziewsky in the subcutaneous tissue of the rabbit, and later by Neufeld in his study of the Pfeiffer phenomenon with the pneumococcus. However much this process appears as a rapid destruction and death of the cells, the following reasons would lead one to believe that this is only apparent and not real:

1. Neufeld has shown that dead pneumococci agglutinate equally as well as the living organisms, and that by simply heating almost to boiling the cocci forming the agglutinated masses in which no organisms can be distinguished again take on normal shape and outline. Their affinity for the ordinary stains also returns.

2. I was able repeatedly to grow the organisms from the masses agglutinated most perfectly, in which no organisms could be demonstrated in stained preparations, almost as well as from the controls in which organisms were abundant. Living organisms were demonstrated as long as twenty-five days after the agglutination tests were made. Further, agglutinated and supposedly dead pneumococci were found to be virulent to rabbits in a number of cases, the pneumococcus being isolated in pure culture from the rabbit's blood after death in each instance.

3. Further, by the use of the plate method with blood agar, I have been able to show that the development of the organisms and their death coincide very closely indeed when grown in normal serum, which remains practically clear, and when grown in pneumonic serum, in which there is an early agglutination and later an abundant sediment. Hence the conclusion drawn by a number of investigators, based upon the microscopic picture, that this granular substance is the result entirely of a rapid lysis of pneumococcus cells, is probably incorrect. (See section on the production of acid by the growth of pneumococci in pneumonic serum.)

Results.—In the total number of 200 tests the serum from 96 cases of pneumonia and 55 strains of pneumococci were used. In the main, the results of the previous investigators have been confirmed. The serum on the second day in one case showed distinct agglutinative properties, agglutinating its own and, more feebly, other pneumococci. Agglutination grows more marked

as crisis is reached, and diminishes gradually thereafter. The serum of one case showed rather marked agglutinative power three weeks after crisis. This case later developed empyema, which was found to be due to the pneumococcus.

Aging the serum, both on ice and at room temperatures, diminishes its powers of agglutination, but my results do not show the rapid diminution spoken of by Neufeld. Marked agglutinating properties were found several times as late as thirty days after the serum was drawn. Bensançon and Griffon have found that occasionally pneumonic serum agglutinated only pneumococci derived from the same patient. While pneumococci and serum from the same patient agglutinate most perfectly, yet in no instance did I fail to obtain agglutination with pneumococci of different strain. The highest dilutions at which agglutinations could be obtained were 1:40 or 1:50. The agglutinin seems to be as abundant in the serum obtained by allowing the blood to clot as in that obtained by immediate defibrination and centrifugalization.

After a careful analysis of my results, I am unable to establish any constant difference in the degree of agglutination in the sera of the fatal and non-fatal cases.

Owing to the almost uniformly positive results of the blood cultures, I am unable to state definitely whether or not there exists any constant relation between the presence of the organisms in the blood and the abundance of the agglutinin. Nor has it been possible to establish any definite relation between the abundance of the agglutinin and the height of the leucocytosis. In a number of instances where there was a high leucocyte count the serum possessed strong agglutinating properties. However, the sera in three of the cases (Nos. 55, 35, and 24), in which the organisms failed to grow in cultures, showed definite agglutination. Yet in smears from the blood the organisms were demonstrable. The serum from two cases of epidemic cerebrospinal meningitis had no agglutinative power over pneumococci, but did agglutinate the diplococcus intracellularis. Erysipelas serum was found to agglutinate the pneumococcus in dilution below 1:20 only.

VI. THE PRODUCTION OF ACID BY THE GROWTH OF PNEUMOCOCCI IN PNEUMONIC SERUM.

Pneumococci grow equally well in normal and pneumonic serum (Table III). In the former it produces scarcely any appreciable precipitate, while in the latter a voluminous sediment appears, often comprising at the end of forty-eight hours one-half of the serum in the test-tube. This precipitation is a distinct reaction, and has apparently nothing to do with the agglutination which occurs very early. That this voluminous sediment is the result of an exceedingly rapid growth and death of the pneumococci, as the picture under the microscope suggests, and as Neufeld, Wadsworth, and others would lead us to believe, seems unlikely, because by the plate method I have shown that the development and death of the cells in this serum follow the same course exactly, as in normal serum. Furthermore, both Neufeld and Wadsworth obtained similar precipitates by adding serum of animals immunized against pneumococci to culture fluids and to pneumococcal suspensions in salt solution from both of which the cocci had been removed.

In trying to determine to what the precipitation so constantly observed in pneumonic sera might be due, the reactions of the sera were taken at intervals after inoculation with the pneumococcus, and it was found that the growth of the pneumococcus in pneumonic serum results in the production of a marked acid reaction (Table IV), while in normal serum the reaction remains alkaline. This has proved to be constant. The reaction as a routine was tested by means of a platinum loop and a good quality of litmus paper. Accurate titrations have been made often enough to prove the efficiency of litmus paper as an indicator. The acidity ranged from 1 to 2.3 per cent. to litmus, and as high as 3 per cent. to phenolphthalein. The acidity develops rapidly and in direct proportion to the rapidity of growth. It is usually quite marked after eighteen hours' incubation, increasing until living micro-organisms are no longer demonstrable; after this time the reaction does not change. The pneumonic sera tested in this way were obtained in all stages of the disease from the third day to one day after crisis. No attempt has been made to isolate the

acids other than to determine that lactic acid is present. Filtering pneumonic serum through porcelain filters does not remove the substance or substances upon which the acid production depends, while dialysis does. By growing the pneumococcus in dialyzed pneumonia serum, the reaction remains unchanged. Keeping the serum in the ice-chest for as long as forty-three days does not change the property of acid production when pneumococci are inoculated. Heating pneumonic serum to 56° and 60° for one-half hour delayed and diminished the acid production in all of fifteen sera so tested, while in five no acid appeared. The presence of red blood corpuscles in the serum does not seem to modify the production of acid.

That the precipitate constantly observed when pneumococci grow in pneumonic sera is due to the production of acids is probably true, for the following reasons:

1. Precipitation is proportional to the degree of acidity present.
2. It is absent in heated serum when the reaction remains alkaline.

TABLE IV.

The Production of Acid by Growth of Pneumococcus in Pneumonic Serum.

NATURE OF SERUM	NO. OF SERA TESTED	REACTION											
		18 Hrs.			48 Hrs.			60 Hrs.			84 Hrs.		
		Alk.	N.	Ac.	Alk.	N.	Ac.	Alk.	N.	Ac.	Alk.	N.	Ac.
Pneumonia	32	3	1	28	..	1	31	32	32
Normal	28	28	28	28	28
Pneumonic—heated to 60° C. for 30 minutes	6	4	..	2	3	..	3	2	..	4	2	..	4
Pneumonic—heated to 56° C. for 30 minutes	9	7	2	..	3	..	6	3	..	6	3	..	6
Pneumonic—dialyzed	4	..	4	..	4	4	4
Pneumonic—filtered	1	1	1	1	1
Scarlet fever	5	5	5	5	5
Scarlet fever—heated to 56° C. for 30 minutes	4	4	4	..	4	4	4

NOTE.—As a rule, 0.5 c.c. of serum was inoculated with pneumococci. Blood-agar plate cultures were made with fixed quantities immediately, after eighteen hours, and after forty-eight hours, and the colonies counted. The number of colonies increased and decreased in all sera in the same manner as is shown in Table III. The pneumococci did not die out more rapidly as the acid reaction appeared in pneumonic and scarlet fever serum than in the normal and the heated sera.

3. A similar precipitate is produced by the addition of an organic acid to the culture in normal serum and in heated pneumonic serum.

4. By restoring the original alkalinity of the mixture a considerable amount of the precipitate is dissolved.

I have found also that pneumococci produce acids in the serum of patients in other febrile diseases than pneumonia, especially in scarlet fever. Here it also produces a sediment similar to that obtained in pneumonic serum, but not so abundant. Here too the pneumococcus may be agglutinated.

Numerous experiments have been made to test the reaction of normal human serum in which pneumococci were grown. A large number of different strains of pneumococci were used. In every case the reaction which was tested at the end of eighteen, twenty-eight, sixty, and eighty-four hours after inoculation remained alkaline. In order to determine definitely that actual growth took place, colonies were counted immediately, at the end of eighteen, and after forty-eight hours, the results in this respect corresponding closely to those given already in the tables.

Streptococci do not produce any change in reaction when grown in pneumonic serum. The same is also true of staphylococcus aureus.

The production of acids by the growth in the test-tube of pneumococcus in the serum of pneumonia patients naturally leads one to raise the question whether this does not also occur in the consolidated lung and in the blood of patients. This is not improbable in view of the facts that when the pneumococcus is grown in extracts of consolidated lungs it produces acids, and that late in fatal cases of pneumonia there is a diminished alkalinity of the blood (von Jacksch). Perhaps some of the toxic symptoms in pneumonia are due to acid intoxication. If this is so, it would explain the good results reported by some from the administration of alkalinizing agents. Unfortunately I have not had the opportunity to study carefully the time of appearance and disappearance of the acid producing power of pneumonic serum, and important questions such as its possible relations to crisis, must be left for future consideration.

VII. HEMOLYSIS IN FATAL PNEUMOCOCCUS INFECTIONS OF RABBITS.

In the course of my experiments attention became directed to the relatively early hemolysis of the blood invariably present in rabbits dead from a rapidly fatal dose of the pneumococcus. Other rabbits dead from other causes than infection, such as traumatism, hemorrhage, chloroform, and ether anesthesia, did not show this. The blood was carefully drawn into small pipettes, the end sealed, and immediately centrifugated to separate the corpuscles from the serum. To be able to express in percentages the degree of hemolysis present, an arbitrary colorimetric scale of solutions of carmine in sealed pipettes of the same diameter was constructed. That post-mortem hemolysis in general is mostly due to the growth of micro-organisms seems likely, because in animals dead from traumatism there is practically no solution of the corpuscles as long as the blood is sterile, but as soon as the blood becomes invaded by bacteria, hemolysis rapidly increases. That the hemolysis of the blood in pneumococcus infection of rabbits is probably due, wholly or in part, to the action of pneumococci seems likely for various reasons. In a number of animals there was a slight hemolysis shortly before death, while the blood was crowded with pneumococci. The pneumococcus, especially when highly virulent, produces an appreciable zone of hemolysis (which is always greenish in color) around the colonies on blood agar. (It may be of interest to note here that the surface colonies produce a greater degree of hemolysis than do the deep colonies; indeed, it is frequently absent in the latter, just as if oxygen were necessary to produce this phenomenon.)

When rabbit's blood is inoculated with pneumococci in the test-tube, the increase of hemolysis seems to run hand in hand with the increase of the pneumococci. This is shown by the plate method in the following experiment, repeated a number of times: Three test-tubes, each containing 1 c.c. of rabbit's blood, were taken, one used as a control, and the other two inoculated with a large number of highly virulent pneumococci. One of these, with the control, was placed in the incubator, and the other one in the ice-chest. At the end of twenty-four hours the tube in the incubator containing the pneumococcus showed a high degree of

hemolysis and multiplication of pneumococci, while the one in the ice-chest: where the number of pneumococci remained stationary, showed no hemolysis. The heat of the incubator alone seems to produce only a slight hemolysis, as shown in the control. Again, similar experiments with the blood of rabbits, dead from the pneumococcus infection, and crowded with pneumococci, show that the hemolysis increases rapidly in the incubator, while the pneumococci rapidly decrease in number, just as if their disintegration liberated a hemolytic substance. Where the blood is put in the ice-chest, the hemolysis, instead of remaining the same as in the former instance, shows some increase. In some cases, as the hemolysis increased, the plates showed a diminution of the number of cocci.

These observations led me to test the hemolytic properties of various filtrates of a highly virulent pneumococcus. Filtrates obtained from bouillon and milk cultures incubated for from twenty-four hours to several days, and from cultures in heated (56° for thirty minutes) and unheated human serum, and in equal parts of heated human and rabbit's serum, showed no hemolysis.

The filtrates of infected rabbit's blood, incubated for twenty-four hours, caused distinct hemolysis of fresh washed rabbit corpuscles in two instances, and a trace in the third. This power was destroyed by heating to 56° C. for thirty minutes.

VIII. DIFFERENTIATION OF PNEUMOCOCCI AND STREPTOCOCCI.

Quite independently of Schottmüller's recent work, I have found that in the use of blood-agar plates we have a seemingly reliable and simple method of differentiating pneumococci from streptococci. The blood-agar plates were prepared by mixing 0.3-0.5 c.c. of sterile defibrinated blood (rabbit or human) to the tube containing 5 c.c. of melted plain agar cooled to 45° C.; it is best not to get more than 300 colonies per plate. Upon this medium the colonies of pneumococci grow larger, are themselves of a distinct green color, and are surrounded by a narrow zone in which the corpuscles are destroyed. This zone is never clear and transparent, but always opaque and of a distinct greenish tinge. The colonies of streptococci, on the other hand, remain small and

grayish, and are surrounded by a perfectly clear transparent zone of hemolysis, the size of which is in direct proportion to their virulence. Sixty-five strains of pneumococci have been tested. These were obtained mostly from the blood: a number of them came from the consolidated lung and from the sputum of pneumonia patients. The results outlined above were constant without exception.

A pneumococcus whose virulence had been increased to such a degree that the intravenous inoculation of 1/1,000 of a loop of broth culture caused death in a rabbit in twenty-four hours produced a wider zone of hemolysis, if we may call it so, than did the organisms of less virulence, but even here the zone was not clear and transparent.

Thirty-five strains of streptococci have been tested. For most of these I am indebted to Dr. Weaver and Dr. Ruediger, and in every instance the colonies remained small and surrounded by a well-marked clear zone of hemolysis. My results are therefore in full accord with those previously obtained by Schottmüller.

The fact that pneumococci produce acid reaction in pneumonic serum, whereas streptococci do not, so far as I have observed, may be mentioned as another point of difference between these organisms.

IX. CONCLUSIONS.

The following conclusions are believed to be justifiable:

1. With improved technic, using for inoculation large quantities of blood, the pneumococcus can be recovered in practically all cases of croupous pneumonia, and in obscure cases of pneumococcus infection blood cultures may be a diagnostic method of positive value.

2. Pneumococcemia in pneumonia does not mean an especially unfavorable prognosis, and is to be regarded here, as in subcutaneous pneumococcus infections of the rabbit, not as an especially ominous or agonal process, but rather as an integral part of the infection.

3. There seems to be a diminution, either in the number or viability, or both, of the pneumococci in the blood at the time of crisis.

4. The number of leucocytes in pneumonia in man and in the pneumococcus infection of the rabbit is an index to the degree of resistance, and the leucocytes probably constitute an important factor in combating the infection. The clinical observation that a high leucocytosis means a favorable prognosis irrespective of the presence of pneumococci in the blood is undoubtedly correct.

5. The hypoleucocytosis developing upon a previous hyperleucocytosis during the course of many fatal cases of lobar pneumonia (and in pneumococcus infection of the rabbit) cannot be looked upon as due to the entrance of the pneumococcus cells into the blood stream, but probably rather as the result of an exhaustion of the resisting powers.

6. The leucocytosis may be incited, at least in part, by soluble substances liberated by pneumococci.

7. It was not possible to establish any appreciable difference in the degree of virulence of the pneumococcus isolated early or late in the course of the disease, nor in the fatal or non-fatal cases.

8. Fresh normal and pneumonic blood and serum have no bactericidal influence upon the pneumococcus. Whatever other differences they may have, so far as this point goes the serum from pneumonia patients behaves exactly as does normal serum.

9. The interesting question whether lobar pneumonia is the primary result of a direct local infection of the lung, or a secondary localization of a primary blood invasion, is as yet hardly ripe for final discussion, but that the latter does occur, at least in some instances, is not altogether unlikely.

10. Agglutination of the pneumococcus by pneumonic serum is constant, but the conclusion drawn by certain investigators that the voluminous sediment and the early clouding of certain immune sera in which the pneumococcus is cultivated are due to a rapid lysis of the cocci seems incorrect. It seems to concern a precipitate the result of acids which appear when pneumococci are grown in pneumonic serum.

11. The production of acids by pneumococci in pneumonic serum suggests that some of the toxic symptoms of pneumonia may be due to acid intoxication. This needs further study.

12. Since the viability and the virulence of the pneumococcus are preserved for a remarkably long period upon blood agar, we have in this medium an important aid to all investigations of this micro-organism. The constituent of the blood which has this rather remarkable effect is probably the hemoglobin. Blood-agar plates are valuable for differentiation of pneumococci and streptococci.

13. That the pneumococcus by its growth in rabbits produces a soluble hemolysin for the corpuscles of this animal is probably true. That this is either small in amount or unstable, or both, is likely.

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EPIDEMIC GONORRHEAL VULVO-VAGINITIS IN YOUNG GIRLS.*

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THE occurrence of gonorrheal vulvovaginitis in young girls has obtained only in recent years the recognition to which its serious consequences entitle it. In addition to the numerous instances in which the infection is acquired in the home by sleeping with other members of the household, there are cases in hospitals, asylums, etc., in which the development, mode of infection, and number of cases justify the designation "epidemic vulvovaginitis." There are obvious reasons why this form of gonorrheal infection has not become widely known.†

The earliest record I have been able to find in which the significance of these manifestations was realized is that of Atkinson,¹ who in 1878, in a report of an epidemic among six young girls in a dormitory of a charitable institution in Baltimore, predicted the discovery of an infectious principle common to vulvovaginitis, purulent ophthalmia, and ulcerative stomatitis. There were two or three cases of purulent ophthalmia in the dormitory, and the disease was probably spread by improper practices. The cases averaged two months in duration.

The epidemic described by Fraenkel² in 1885, affecting sixty-two girls in the Hamburg hospital and lasting for four years, occurred at a time when there was still considerable discussion concerning the etiologic relation between the gonococcus and vulvovaginitis in children. On account of the few complications, Fraenkel believed that the diplococcus obtained in cover-glass preparations and cultures from these cases was not the gonococcus of Neisser, but a closely related organism. Conjunctivitis appeared in only

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† In reviewing the literature, a number of articles were found dealing with statistics gathered from dispensaries, or with cases admitted to hospitals with a pre-existing discharge; these were not included in the following summary.

four of these cases, and in a few inoculation experiments made by implanting the organism upon the conjunctivæ of three practically moribund girls, a very mild inflammation was produced in one case, and in the others death occurred before results were noted. The epidemic was confined mainly to patients with scarlatina, all of whom were in one building, although cases appeared in other and separate buildings. The mode of transmission was ascribed to the use of common bath-tubs and closets.

In the same year Czeri³ and Lennander⁴ reported epidemics and, in contrast to the view adopted by Fraenkel, declared positively that the organisms in their cases were gonococci. The first of these occurred in Buda-Pesth, and Czeri found the organism by cultures in all of the twenty-six cases involved. This epidemic had its origin in the admission to the hospital of a case of vulvovaginitis. The infection of other children was ascribed to contaminated clothing (bed-linen), lavatories, instruments, bandages, and to mutual contact. A nurse in charge of one of these cases developed a purulent ophthalmia and panophthalmitis, for which removal of the eye was necessary. The cases examined by Lennander numbered eighteen, and the epidemic developed in the children's hospital in Stockholm from the admission of a girl with measles who also had a vaginal discharge.

In the following year an epidemic occurred in the Hebrew Orphans' Home in New York city;⁵ thirty-five girls were affected, and eighteen developed purulent ophthalmia. Its spread followed the introduction of four girls with gonorrheal vulvovaginitis.

Following the admission of cases of vulvovaginitis to the children's hospital in Heidelberg, Dusch⁶ noted the development of nine other cases, one in 1886 and eight in 1888.

The bacterial nature of the infection was not so definitely determined in the epidemic in the children's hospital of Paris reported by Ollivier,⁷ although it undoubtedly belongs to this category. Fifteen became infected following the entrance of two girls with vulvovaginitis.

A similar absence of accurate bacteriological observations obtains in the report by Suchard,⁸ in 1888, of two epidemics

occurring in a hospital at Lavey, where hydrotherapy was probably responsible for the transmission. In each epidemic the infection spread among a group of patients newly arrived from Paris and assigned to a common bath. In one instance, out of thirteen using the bath, eleven patients (all girls) were infected, those escaping being a boy, and a woman of thirty; in the other group the vulvovaginitis was discovered in all of the twelve girls using a freshly cemented bath.

The importance of public baths and common lavatory utensils in spreading infection is well shown by the frequent references in the literature to the report by Skutsch⁹ of an extensive epidemic of gonorrheal vulvo-vaginitis in Posen in 1891. Two hundred and thirty-six cases developed among girls using the free baths, and many suffered from a complicating purulent ophthalmia. Boys using the same baths escaped the infection.

There are two reports in 1895: one by Fischer¹⁰ from the children's hospital at Altona, in which forty of the fifty-four cases analyzed were instances of infection in the hospital; the other a report by Weill and Barjon,¹¹ in which careful study of the conditions led to the assumption that the infection was spread by the thermometer used to take the temperatures per rectum. Following appropriate methods of disinfection of this instrument, the epidemic promptly ceased. The cases in this epidemic number twenty, and all appeared during the three months subsequent to the admission of a girl of five years with vulvovaginitis.

A second epidemic in the Hebrew Orphans' Home in New York city, affecting sixty-five girls, occurred in 1896, and is reported by Sheffield.¹² All the cases appeared in the month following the entrance of a patient with gonorrheal vulvovaginitis and ophthalmia. Transmission was attributed to the custom which prevailed of bathing twenty to thirty girls in one large bath simultaneously. From a number of cases growths of the gonococcus were obtained on serum-agar. Of complications, localized peritonitis developed in four cases, synovitis of the ankle in two, and ophthalmia in six.

A careful review of the cases of this form of gonorrheal infection observed in the children's hospital in Zurich between the

years 1874 and 1895 has been made by M^{me} Skiba-Zaborowska.¹³ For six years (1877-82) the disease was epidemic; during this period fifty-one cases were observed, only nine of which, entering during the first two years, were diseased at the time of entrance. During subsequent years, beginning in 1887, the isolation of all children entering with a vaginal discharge prevented spread of the disease among resident cases.

Less extensive epidemics have been reported by Cnopf¹⁴ from the children's hospital of Nuremberg in 1898, and by Koplik¹⁵ from New York in 1903. The ten cases observed by Cnopf were infected from other infants with ophthalmia, and a similar number noted by Koplik followed directly upon the admission of boys with gonorrheal proctitis. In discussing the modes of transmission, Koplik especially emphasizes the danger of insufficient boiling of bed-linens.

A recent article by Kimball¹⁶ concerns chiefly a series of eight cases of multiple gonorrheal arthritis or bacteriemia (seven males and one female, all under three months) without demonstrable portals of entry; the gonococcus was identified in the arthritic fluid microscopically in all the cases, and by cultures in six. Mention is also made by Kimball of seventy cases of vulvo-vaginitis in the Babies' Hospital of New York, 90 per cent. of which were infected in the hospital. These cases appeared as a series of small epidemics, each following the admission of a child carrying the infection.

In a review of the literature by Dr. I. A. Abt,¹⁷ mention is made of an epidemic in St. Anna's Children's Hospital, Vienna, in which transmission was due to unclean thermometers.

In the nineteen epidemics mentioned in the foregoing there are reported 690 cases of gonorrheal vulvo-vaginitis, the largest epidemic being that described by Skutsch at Posen and involving 236 girls. In twelve epidemics, comprising 498 cases, the age limits range from three months to fourteen years, the average ages being nine years for 235 cases, seven years for 59 cases, and five years for 144 cases. The source of the infection in eight of the epidemics (Lennander, Sheffield, Leszynsky, Dusch, Czeri, Weill and Barjon, Ollivier, Kimball) was traced to the admission

of one or more girls with a pre-existing vaginal discharge; in two (Koplik) the outbreak followed directly upon admission of a boy with rectal discharge. In three (Cnopf, Atkinson) the infection spread from cases of gonorrheal ophthalmia. In the remaining six epidemics the source of infection could not be ascertained. In some of the epidemics the modes of transmission have been positively ascribed to: common baths (Suchard, Skutsch, Sheffield); manipulations by the children (Dusch, Atkinson); thermometers (Weill and Barjon); nurses' hands (Cnopf); baths and closets (Fraenkel). In other epidemics transmission has been attributed to: towels and wash-rags, diapers, bandages and bed-linens, instruments, lavatory fixtures, and the children's hands. Kimball, Koplik, and Ollivier lay especial emphasis on the danger of transmission by the hands of nurses.

The statements of most observers are to the effect that the disease in individual cases is of rather long duration. In 205 cases the average duration was stated to be two months. M^{me} Skiba-Zaborowska in her series of 52 cases refers to a duration of "months," many cases resisting all treatment. Fischer also records some cases as uncured after six months. Kimball has called attention to the difficulty with which the vulvo-vaginitis is cured in children over one year, in contrast to the more prompt recovery in younger infants.

The determination of the gonorrheal nature of the discharge was based on microscopic examination in 240 cases (Weill and Barjon, Cnopf, Skiba-Zaborowska, Skutsch, Dusch, Lennander), and on microscopic examination with cultivation on nutrient media in 91 cases (Sheffield, Czeri). As before stated, Fraenkel regarded the organism in his 62 cases as a distinct, though related, organism, because of clinical differences in the diseases. In view of present knowledge, this may be considered as the gonococcus.

The records of the earlier observers indicate that very little attempt has been made to establish the gonorrheal nature of secondary infections of other parts of the body. In seven epidemics, comprising 477 cases, in which such investigations were made, the frequency of the complications mentioned is set forth in the following table:

Ophthalmia	-	-	-	34	Sheffield, Leszynsky, Fraenkel, Kimball, Skiba-Zaborowska
Urethritis	-	-	-	20	Fischer
			Frequent		Skutsch
				1	Skiba-Zaborowska
Infection of glands of Bartholin	-	-	-	13	Fischer
Arthritis	-	-	-	5	Kimball, Sheffield, Skiba-Zaborowska
Peritonitis*	-	-	-	5	4 local (Sheffield); 1 fatal—1878 (Skiba-Zaborowska)
Proctitis	-	-	-	2	Sheffield
Endometritis	-	-	-	1	Sheffield

The variety of complications presented above is easily supplemented from the numerous articles bearing upon the secondary infections by the gonococcus as follows: Cystitis is considered not uncommon by various writers,¹⁸⁻²⁰ though one²¹ takes an opposite stand. Endometritis, salpingitis, oöphoritis, and peritonitis are lamentably frequent occurrences,²² the three last mentioned generally being associated²¹ and often terminating in sterility.²³ It is probable that further study of this variety of infantile vulvovaginitis will reveal cases in which gonorrheal stomatitis occurs as a complication. Kimball suspected that the mouth was the point of primary infection in three of his eight cases of multiple gonorrheal arthritis.

The possibility of some relationship between epidemic vulvovaginitis and pre-existing acute infectious diseases, such as scarlatina, measles, and typhoid fever, has been pointed out by Fraenkel, Dusch, Cnopf, Weill and Barjon, and is explained by Cnopf on the basis of an increased susceptibility of the children.

The hospital epidemic of gonorrheal vulvovaginitis reported here extended over a period of thirteen months, and its study was undertaken at the suggestion of Dr. E. R. Le Count.

This epidemic involved eighteen girls and one boy. During the thirteen months there were three periods, of about two months each, in which no new cases appeared, but, as will be shown, each interval was bridged over by resident cases, so that transmission was never completely interrupted. On July 21, 1902, a boy

*Twenty-two other cases of peritonitis in young girls are reported in the literature as of gonorrheal nature; in all the primary infection was non-venereal.

(Case I), aged two years, was admitted to a general ward for rectal abscess and anal fistula, which was operated on twice during the succeeding months. Seventeen days after admission edema of the penis, with a urethral discharge, containing diplococci, was noted; eight weeks later (October 5) the discharge had ceased, and the case was considered cured. Three days from this date (October 8) Cases II and III, sisters aged three and six years respectively, were admitted to the same ward for typhoid fever. In the younger of these (Case II) there appeared, two days after entrance, a vaginal discharge containing diplococci refusing Gram's stain; and during the ensuing twenty-one days a similar discharge was noted in five other girls (Cases III-VII): in one, after twelve days; the next, after fifteen days; the elder sister (Case III), after sixteen days; and the two remaining, on the twenty-first day. Three of the patients had typhoid fever, one genu-valgum, one congenital dislocation of the hip, and one tuberculous spondylitis.

The attempts made to prove the gonorrheal character of the infection in these cases included the usual methods of examination of cover-glass preparations. Cultures were also made from two cases upon ordinary media and upon ox-blood serum and agar coated with human blood, which yielded only negative results as regards the gonococcus. These six cases were then removed to a separate ward, and the progress of the epidemic was delayed two months by this procedure. Three weeks before the appearance of any new cases there was admitted to a room adjacent to this isolation ward an infant (Case VIII) under treatment for cleft palate. This propinquity was due to the fact that the child's mother was a patient in the same room. It has been ascertained that the temperature of this infant was taken per rectum by the same nurse who was taking the temperatures of the cases of vulvo-vaginitis. Following the removal of this infant (Case VIII) to a general ward, purulent vaginitis appeared in another infant (Case IX) occupying the same bed.* These facts led to a more careful examination of Case VIII, in whom gonorrheal vulvovaginitis

* It was afterward learned that a vaginal discharge was discovered by the mother of another child (Case X) who had left this general ward a few days previously.

had been previously suspected on account of the odor, and the suspicion was confirmed. Eleven and fourteen days later two other infants in the same ward (Cases XI and XII) developed purulent vaginal discharges. In one of these four cases measles appeared, and they were all quarantined; so that at this time there were in the hospital two isolation wards, containing altogether eight cases of vulvovaginitis, two of the first group having been discharged.

Two months later one of these patients, with the vulvovaginitis still persisting, developed scarlatina, and was transferred to the Memorial Institute for Infectious Diseases. There had been admitted to the Memorial Institute a few days before a child (Case XIII) who, during the previous three weeks, had been in the general ward in the hospital. The morning following her transfer she was found to have a vulvovaginitis with a mucopurulent discharge, containing diplococci negative to Gram's stain and non-cultivable on ordinary media. In about two weeks following the entrance of these two sources of infection vulvovaginitis appeared in two other girls (Cases XIV and XV) convalescent from scarlatina; but no other cases developed in the Institute.

Of this third group of cases one was removed by the relatives before the vaginitis was cured, and the other three were transferred to the single ward in the hospital, which at this time contained the remaining cases of vulvovaginitis. These cases were discharged after varying intervals, until there remained but two patients—one a typhoid-fever case, and the other a case of congenital dislocation of the hip. During the month of May the latter patient was frequently dressed in clinics, and on two occasions was conveyed to and from the clinic on the same hospital cart with several other girls, also surgical cases, from the general ward for children. Within one week (June 3) vulvovaginitis was observed in one of these girls (Case XVI); five days later there were two more cases (Cases XVII and XVIII); and four days after this an additional case (Case XIX)—all in the same ward. The four new cases were isolated with a special nurse, and were attended by a physician who saw no other cases in the hospital.

The last of these cases was discharged after ten weeks, and since then no new cases have appeared.

Of the nineteen* cases included in this epidemic, seven were medical cases—five typhoid, one scarlatina, and one epilepsy. The surgical cases included three each of cleft palate and tuberculosis spondylitis, two cases of congenital dislocation of the hip, and one each of genu valgum, angioma of the face, tuberculosis of the ankle and rib, and anal fistula. All were girls, except the case first mentioned (Case I); their ages ranged from six months to thirteen years. The following table shows the ages of the individual cases:†

6 months	-	-	-	-	-	-	1
8 "	-	-	-	-	-	-	1
2 years	-	-	-	-	-	-	2
3 "	-	-	-	-	-	-	7
4 "	-	-	-	-	-	-	2
6 "	-	-	-	-	-	-	3
9.5 "	-	-	-	-	-	-	1
10 "	-	-	-	-	-	-	1
13 "	-	-	-	-	-	-	1
Total	-	-	-	-	-	-	19

As has been shown by the consideration of other epidemics (Fischer, Skiba-Zaborowska), this form of infection is very resistant to treatment. The eight cases discharged as cured had an average duration of 122 days, the extremes being 39 and 254 days respectively. It is worthy of mention that of these long-standing cases four had typhoid fever; two congenital dislocation of the hip, wearing plaster casts for a considerable time; one case had tuberculous spondylitis, and one anal fistula. Six cases left the hospital uncured, and in two others the cure was questionable at the time of their discharge. One case died of bronchopneumonia following measles, another of tuberculous meningitis, and in the remaining case the infection was not discovered until after the patient had been sent home.

*This number is equivalent to 6 per cent. of the total number of children under fourteen years in the hospital during this same period, and 12.5 per cent. of the total number of girls.

†The average age of all the patients under fourteen years in the hospital during the epidemic was seven years and two months.

Some of the patients presented symptoms pointing quite definitely to infection of other parts of the body with gonococci. The most interesting case illustrative of this is Case XIV. This patient, aged ten years, was admitted for cleft palate, and, following the development of scarlatina, was removed to the Memorial Institute. During desquamation a vaginal discharge, and pains in the elbows and knees, appeared on the same day. There was slight swelling about the joints, with extreme tenderness. These symptoms were ushered in by a continued fever, which lasted eleven days, averaging 101.5° F., with one remission to normal on the second day. The involvement of the joints, diagnosed as multiple gonorrheal arthritis, gradually diminished in severity, and disappeared after eight days. A few days later the temperature rose to 103° F., the patient complained of severe abdominal pain, and there was marked tenderness in the hypogastrium, with tympany, constipation, vomiting, and a leucocytosis of 17,000. These acute symptoms were diagnosed as due to gonorrheal peritonitis; they lasted only a few days, but abdominal pain and tenderness, with an occasional slight rise in temperature, remained for approximately four weeks.

Only one case developed a severe and persistent conjunctivitis, and this on microscopic examination showed only streptococci. In three other cases there was a mild conjunctivitis, which was not examined. Two other complications—suppurative otitis media in five cases, and axillary abscess in one—are to be regarded as more closely related to the measles and typhoid fever which were complicated by the vaginitis, than to the gonorrheal infection.

As to the source of the epidemic, it will be remembered that the first case of vulvo-vaginitis (Case II) was discovered two days after her admission, and five days after a boy (Case I) in the same ward had been pronounced cured of gonorrheal urethritis. The indications point toward the former of these as the agent by whom the infection was introduced. Yet the short duration of the period of incubation* of gonorrhea in young girls renders it possible that the girl (Case II) became infected after entrance by transmission from the boy.

* Incubation in males ranges from three to seven days; in females, from two to five days, and is especially short in young girls (Keyes, Morrow, Schmidt, and Pedersen).

Careful examination of the conditions surrounding these patients reveals very few possible modes of transmission of the infection from case to case that have not already been considered by other writers. There is little doubt but that the first period of intermission during which no new cases developed, was the result of careful isolation. That this period was limited to two months is explained by the circumstances, already described, under which the temperature of Case VIII was taken. During May, 1903, no new cases were observed. During the latter part of this month, Case VI was twice dressed at the clinic for the congenital dislocation of the hip, with which she was also afflicted. She was conveyed to and from the clinic upon a hospital cart with other girls, and it has seemed probable that transmission was effected either in the clinic or upon the cart. The necessity for complete isolation to prevent spread of the infection was not realized during the earlier months of the epidemic, as has been pointed out, and with the inauguration of radical measures the outbreak was soon brought under complete control.

In this epidemic, as in several found in the literature, there is a relationship to pre-existing infectious diseases. Five cases of typhoid fever and three cases of scarlatina developed the vulvo-vaginitis. Two other cases, one of scarlatina and one of measles, appeared two months and four days, respectively, after the vaginal discharge had been found,

Bacteriologic investigations during the progress of the epidemic yielded the following results: a diplococcus regarded as the gonococcus was found in cover-glass preparations from ten cases; from five other cases the organism failed to grow on ordinary media, although cover-glass preparations demonstrated its presence;* and finally it was obtained in pure culture and identified as the gonococcus from three cases, the others having been discharged when its cultivation by the writer was undertaken.

The medium employed was a mixture of plain agar condensed to two-thirds its bulk, with either hydrocele, pleuritic, or ascitic fluid, in the proportion of 2 to 1, and solidified, slanting in test-tubes. The transudate was gathered in sterile flasks, distributed

* For the daily examination of cover-glass preparations from a number of cases, after thorough isolation had been instituted, we are indebted to Dr. T. O. Greig.

in sterile test-tubes, and incubated twenty-four hours at 37° C. to determine its sterility. It was then added to agar at 45° C., each tube being prepared separately; the transudates were never subjected to a heat of over 50° C.; thus prepared the tubes were sealed with rubber caps sterilized by a solution of HgCl_2 (1:1,000), slanted until solid, and incubated at 37° C. for two days to again determine sterility. For isolation the slants were used as plates, and a loopful of the secretion was taken directly from the patient, being spread over the medium by means of the water of condensation.

The first cultures made from four patients yielded only mixed colonies, a bacillus and diplococci from two cases, and a bacillus and staphylococci from the other two. The diplococcus resembled the gonococcus in morphology and staining. The bacillus, which took the ordinary stains and Gram's stain, resembled the vaginal bacillus of Döderlein.* Attempts made on the following day to isolate the diplococci by subcultures proved futile; the same mixed colonies were found, with fewer diplococci.

Attempts were then made to obtain the gonococcus from the case presenting the largest amount of purulent discharge (Case XVI), but again the colonies were mixed, consisting of staphylococci, diplococci, and a few pure colonies of streptococci. In order to obtain a more complete dissemination of the organisms, Petri dishes were substituted for slanted test-tubes, but with no better results. The reaction of the hydrocele-agar was then tested and found to be satisfactory (1 per cent. acid to phenolphthalein). Sample tubes were inoculated with streptococcus and pneumococcus, and both organisms grew luxuriantly.

Meanwhile each patient had been getting a douche with 10 per cent. protargol every four hours four times daily. On July 20, after douching Case XVI with the protargol, she was given a douche of distilled water, and cultures were taken four hours later. These showed no growth after twenty-four hours, but after forty-eight hours numerous pure colonies of diplococci, like the gonococcus in shape and staining reactions, and a few pure colonies

* This organism had been found previously in four cases by Messrs. A. H. Harms and D. M. Green, in the spring of 1903.

of staphylococci were found. After seventy-two hours subcultures of the diplococcus colonies were made on hydrocele-agar and glycerin agar. Growth on the former was luxuriant and presented the following appearances after twenty-four hours: the colonies were round, raised, moist, about 1 mm. in diameter, opaque, and bearing a striking resemblance to ground glass; the fused growth along the stroke was flat, heavy, with straight edges, faintly translucent, and presented the same ground-glass appearance; the inoculated glycerin-agar slants never showed any growth. This culture was maintained on the mixed medium over three months, the organism in several instances being found alive after twelve days' incubation in one tube, as shown by growth after transplantation.

Attention was now turned to Cases XI and XVIII, Case VI having been discharged in the interval as cured. After several unsuccessful attempts to isolate the organism under varying conditions, the method which succeeded in Case XVI was used; the medicated douche was immediately followed by a douche of distilled water, packing being omitted, and cultures were taken after four hours; twenty-four hours later the slants showed many opaque, white, and some paler pinpoint colonies; after forty-eight hours the paler colonies were found to consist of diplococci with the morphology and staining reactions of the gonococcus; they were then isolated on the other slants, and their nature and purity determined.

It has been thought desirable to describe the technic of cultivation at this length because most of the recent text-books give few details, and the methods recorded by Finger, Ghon and Schlagenhauser,²⁴ Steinschneider,²⁵ and others are too cumbersome.

Although the gonococcus has been isolated from only a few cases in this epidemic, the present writer having undertaken its study only during its latter months, it seemed proper to emphasize here the importance of cultivation as an additional step in the diagnosis of gonorrheal infection.

Two other diplococci identical with the gonococcus in morphology and tinctorial reactions, including negative results by Gram's method, have been isolated from male urethras by Stein-

schneider and Galewsky²⁶ in 4.65 per cent. of 86 cases, and can be differentiated from Neisser's organism only by cultural tests, both growing readily on ordinary media.

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A CRITICAL STUDY OF THE METHODS IN CURRENT USE FOR THE DETERMINATION OF FREE AND ALBUMINOID AMMONIA IN SEWAGE.*

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INTRODUCTION.

THE sanitary analysis of water has engaged the attention of chemists for many years, and has been developed to a high degree of perfection. It aims to show the present condition and past history of the water in question, and, in spite of the fact that certain of its most important determinations are of comparative value only, its results, in the hands of a competent chemist, are of no uncertain value. Unfortunately perhaps for the cause of sewage disposal, the methods so long in use for water analysis have been adopted, almost without change, for the examination of sewage and of the products of sewage-disposal works. The analysis of a sewage or effluent of a sewage filter is not undertaken for the purpose of determining the past history of the sample or its present fitness as a drinking water. The problems to be solved are engineering problems. It is gratifying, therefore, to note that the engineer is appreciating more and more the fact that the data upon which he must depend in designing a purification plant are largely chemical.

The question is often asked: "Can you not give us more information on this matter? We wish definite figures which can be used in the compilation of formulæ so that the design of a sewage-disposal plant will be as definite a problem as the construction of a bridge." In fact, the engineering demands have been so exacting and the chemical data so unreliable that discredit has been thrown upon much of the chemical work.

We must frankly confess that we cannot, with our present knowledge at least, give any such absolute information; and it is

* Read at the meeting of the Laboratory Section of the American Public Health Association, October 26, 1903.

almost a matter of certainty that the engineer can never solve his problem in this manner.* The laws of stress and breaking strength are fixed, and capable of experimental determination and of exact application. The sewage problem is never twice the same. Analyses of the sewages of two communities, apparently the same in many respects, show great differences. Climatic conditions exert the greatest influence. The presence of manufacturing wastes in the sewage complicates the problem, and even the quality of the water supply and the geological formation of the region may alter the conditions profoundly. It is on account of these variations that the problem cannot be handled by rigid mathematics. The variables are so many that probably no amount of experimentation could ever supply the necessary constants for use in an engineer's formula.

But while it is true that we cannot give all that is desired in the way of analytical data, there seems to be a growing conviction in the minds of many that our present methods of chemical analysis are inadequate, if not actually misleading at times, and that the time has come for a revision of the methods now employed, and possibly a recasting of the whole scheme of sewage analysis, taking it out of the old familiar ruts, into which it seems to have fallen, and raising it more nearly to the plane of modern engineering requirements.

What, then, should the chemical analysis of sewage include, in view of its functions as indicated above? The analysis should, first, show the total amount of organic matter to be dealt with in the course of twenty-four hours of an average day, and, if possible, the amount of variation from this average day's result to be expected during the year. Next, the nature of the organic matter must be known; that is, whether it is nitrogenous, or carbonaceous, etc. The amount of oxygen available in the sewage, either as dissolved gaseous oxygen, or as nitrate or nitrite, is another important element in the analysis. If the sewage is strongly acid or alkaline, the fact is of importance; but in the ordinary slightly alkaline domestic sewage the determination of alkalinity is of little

*Mr. W. D. Scott-Moncrieff¹ has recently proposed a standard apparatus for use in determining certain constants in sewage. The use of such constants, however, does not seem to have met with favor in England.

moment. The determinations of chlorine, iron, phosphorus, and other mineral ingredients give no information not otherwise obtainable, except in connection with precipitation works, when any abnormal amounts of a mineral substance may be of a considerable importance. A knowledge of the total mineral matter may be of value in certain processes.

The analysis of sewage is made, in short, to give the engineer information to assist him in the design of a purification plant. By comparison of its figures with those from plants already in operation, it is possible to obtain some information as to the probable results to be expected by treating the sewage in question. Such comparisons are often misleading, however, and cases might be cited in which two sewages, agreeing very closely in chemical analysis, yield widely differing results after similar treatment.

The information above outlined as desirable in a sewage analysis is obtained, in water analysis, by the determination of the free and albuminoid ammonias, nitrates, nitrites, oxygen consumed (or absorbed), oxygen dissolved, alkalinity, and fixed solids. It has been assumed that the same methods applied to sewage will give the same kind of information. That this is not necessarily the case I hope to show in this paper, and that I am not alone in distrusting the analytical results as now obtained will be shown by a quotation from a recent paper by Mr. G. W. Fuller.² Mr. Fuller says:

It seems to the writer that the time is ripe for a thorough review and comparison of methods for determining organic matter in sewage on a scale as comprehensive as that made in connection with natural waters under Professor Mallet for the National Board of Health over twenty years ago. To the thought of the writer, the future of sewage chemistry cannot depend upon the question of convenience of manipulation in the laboratory, or that of the degree of skill of the laboratory assistants.

And again:

As to the part played by analytical data in the development of matters of sewage disposal, their value has unquestionably been great. In the opinion of the writer, however, methods employed for studying the composition of sewage in its various phases have fallen somewhat into a rut. It seems questionable whether the amount of added knowledge which may be obtained from a continuance of present methods year after year is going to be commensurate with the cost. In fact, it seems that the time is approaching

when it is worth while to consider a recasting of the program for sewage analysis. This may be thought to be unwise, because future data would not be comparable with the past. This objection is readily met by stating that there should be omitted in the future no determination of value. In fact, it is more complete data which are now needed.

The work here presented is the first portion of a study which will ultimately cover the whole field of sewage analysis. It was carried out at the Sanitary Research Laboratory and Sewage Experiment Station of the Massachusetts Institute of Technology. The sewage used is a representative domestic sewage, coming from the large trunk sewer of the Boston Main Drainage. In planning and carrying out the work, the attempt has been made to show by decisive experiments just what a given determination shows us, to what errors it is liable, and what modifications, if any, are necessary in order that the determination shall yield the best results both from the chemical and from the engineering points of view.

THE FREE AMMONIA.

In the determination of the free ammonia as ordinarily carried out a measured quantity of the sewage is diluted with water, made alkaline with sodium carbonate, and subjected to indirect distillation with live steam. The ammonia is determined in the distillate by the Nessler process. In this procedure we have to consider errors in the distillation and those in the reading. The former might occur in two ways: (1) the whole of the free ammonia present might not be recovered in the distillate; and (2) substances might be decomposed during the distillation which would give off an additional amount of ammonia.

A number of determinations of the ammonia in solutions of ammonium chlorid of known strength have shown that the whole of the free and saline ammonia is recovered, the small errors observed being about equally distributed above and below the true value, and being easily explained by other errors in the process.

On the other hand, it has been found during the present study that a large number of proteid substances are partially broken down during the distillation with sodium carbonate, giving too high a value to the ammonia readings. Among the pure sub-

stances tested in this way the following gave positive results as shown, expressed as percentages of the total nitrogen given off as free ammonia:

Substance	Percentage of Total Nitrogen Found as Free Ammonia
Urea - - - - -	2.0
Gelatin - - - - -	1.4
Casein - - - - -	0.68
Egg albumen - - - - -	3.2
Peptone (Witte's) - - - - -	1.4
Naphthylamine - - - - -	0.1

That this error is of great significance in the analysis of sewage is shown by an extended study on the sewage itself, and on the effluents of sewage purification processes. About fifty samples were analyzed in the regular way, distilling off the free ammonia with sodium carbonate, and then adding alkaline permanganate of potassium and distilling the albuminoid ammonia. A second sample was, in each case, filtered through paper and treated in a similar manner. A third sample was treated with an excess of sodium hydrate and a few drops of a 10 per cent. solution of copper sulphate, and allowed to settle, the clear supernatant liquor being then diluted and nesslerized directly for free ammonia. With very few exceptions the values obtained were lowest in the case of the direct reading, and highest with the unfiltered sample, the filtered sample generally lying between the other two. Moreover, the excess of ammonia found in the distilled sample over that from the direct reading was in all cases roughly proportional to the amount of albuminoid ammonia in the former. The average of fifty such analyses is: raw, 18.8 parts per million; filtered, 17.5 parts; direct, 16.6 parts; albuminoid ammonia—raw, 2.8 parts; filtered, 1.9 parts. The average excess of the free ammonia in the raw samples over that by the direct reading is thus 13.2 per cent. of the direct reading.

Experiments will be presented later in this paper showing that the results obtained by this direct process are much nearer the true values than any others. The subject is brought up at this time merely to call attention to a serious error in the determination as practiced at present.

Passing now to a consideration of the errors in reading, we find here many opportunities for inaccuracies.

Temperature.—Attention has been called by Hazen and Clark³ to the fact that the ammonia standards and the distillates must be at the same temperature to secure correct readings. As long as the regular standards, made up each day, are used, the usual practice of allowing the distillate to stand over night was sufficient to secure this condition. But with the advent of permanent standards the question of temperature becomes one of importance. It has been found that the standards themselves remain practically constant in color during any changes in temperature that may occur in the laboratory. Therefore, if accuracy is to be had in reading ammonias against these standards, the reading must be done at the temperature at which the standards were originally made to compare with the ammonia standard solution, or a temperature correction must be applied.

Dividing the tubes for reading.—When 10 c.c. are taken for an analysis, an amount necessary in order to get satisfactory reading of the albuminoid ammonia, the amount of free ammonia coming off in the first tube is always too high for direct nesslerization. It is customary to allow the tubes to stand over night, and the next morning to take out a suitable quantity, generally 10 c.c., and dilute to 50 c.c. for nesslerization. Samples taken from the top and bottom of several such tubes gave identical readings, showing that diffusion was ample in the time allowed, about fifteen hours. If the tubes are read directly after distillation, however, there is a possibility of error at this point, which must be guarded against by a careful mixing of the contents of the tube.

Variation in the diameters of the nessler tubes.—Considerable variation is met with in the diameters, and consequently in the lengths, up to the mark, of the nessler tubes, as they are found in the market. Our laboratory stock, purchased at two different times from the same dealer, was looked over, and tubes having the maximum and minimum lengths were selected for a comparison. Readings of the same solution with these two tubes gave values of 3.4 and 3.9 c.c. of ammonia standard, respectively—a variation of over 10 per cent. The tubes were extreme cases, it

is true, but the stock included tubes of all lengths between. The only way, obviously, to correct this error is to throw out all tubes which in length do not agree sufficiently well among themselves to give results of the desired accuracy. For very accurate work the plan suggested by Fowler is to be recommended; that is, to select a set of tubes for the standards which shall be all of exactly the same length, and have one extra tube of this length for the readings. All distillates are poured into this tube before reading. This plan is somewhat cumbersome for routine laboratory work, but is the only one which will give strictly accurate results, unless a sufficiently large number of tubes can be obtained of the same diameter.

Color in the glass.—The writer has seen tubes of such a poor quality of glass that a color amounting to 0.3 c.c. of ammonia standard was imparted to the distillate. Such tubes should never be used, since the variation in the thickness of the glass will give a corresponding variation in the color, and the readings will be in error by this amount.

A METHOD FOR THE DIRECT READING OF THE FREE AMMONIA.

Considerations previously given concerning the formation of free ammonia during the distillation indicate the desirability of some method for determining this important constituent in a more direct and more accurate manner.

Farnsteiner,⁴ and his associates at the Chemical and Hygienic Institute, have proposed such a method, as follows: The sewage is treated with sodium hydrate solution and allowed to settle. A portion of the supernatant liquor is removed and nesslerized directly. In the presence of hydrogen sulphide a few drops of a solution of zinc acetate is added.

After a trial of different combinations of precipitants, I have decided upon the following method as best suited to all-around work at a sewage experiment station.* The solutions required are

1. A 10 per cent. solution of copper sulphate.
2. A 10 per cent. solution of lead acetate.
3. A saturated solution of potassium hydrate.

*This successful working out of this method is largely due to the careful work of Mr. G. R. Spalding, research assistant at the Sanitary Research Laboratory.

Fifty c.c. of the sewage to be tested are mixed with an equal volume of water, placed in a short nessler tube about six inches long, and a few drops of the copper solution added. After a thorough mixing, 1 c.c. of the potassium hydrate solution is added and again thoroughly mixed. The tube is then allowed to stand for a few moments, when a heavy precipitate falls to the bottom, leaving a colorless supernatant. An appropriate amount of this solution, usually 2-5 c.c., is then taken, made up to 50 with best water, and nesslerized. We have varied the procedure of Farnsteiner in substituting lead acetate for the zinc salt which he used, and in precipitating a diluted sewage instead of the straight sample. The lead acetate, we find, is a better precipitant, especially for our septic tank effluents, where there is a large amount of hydrogen sulphide present, in which case a few drops of the lead acetate solution is added with the copper solution. The sewage is diluted before precipitation to prevent loss by absorption, and because we find that some effluents are so high in ammonia that there is a noticeable escape of the gas on adding the caustic. Our strongest effluents, however, showed no escape of ammonia, on testing with hydrochloric acid, when properly diluted as described. As to precipitants, no general method can be laid down at present for all cases, but, with a little practice, it is comparatively easy to get the right combination of the three solutions mentioned to bring about the best results.

It has been stated previously that this method yields results nearest to the true ammonia value. The evidence for this statement is as follows: (1) The results were invariably lower than those from the distillation processes. Moreover, when raw sewage and sewage filtered through paper were distilled, the filtered sample in nearly every case gave a result lying between that of the direct reading and that of the raw sample distilled. An average of fifty such analyses gave, for the direct reading, 16.6 parts per million; for the filtered sample, 17.5 parts; and for the raw sample, 18.8 parts. It appears from this that the increase in the values obtained for the distilled samples is due to a decomposition of the nitrogenous matter of those samples, and the increase is about proportional to the amount of albuminoid ammonia given in the

two cases. (2) Experiments with pure nitrogenous substances, likely to be found in sewage, show that they do give off free ammonia on distillation with sodium carbonate. I have given a list of the substances used and the results obtained. As an amplification of this experiment an artificial sewage was made up of peptone, urea, albumin, vitelline, casein, and salt. The ammonia was determined by direct reading, and found to be nil. Enough ammonium chloride solution was now added to bring up the free ammonia to 10 parts per million, and a sample was distilled, while a second sample was precipitated and read directly. The direct reading was 10 parts, while the distillation gave a value of 10.7 parts. That the difference is not so great as is observed in sewage is due to the fact that in the latter we have exceedingly unstable compounds, bodies which are already in the process of conversion to free ammonia, and these are more readily broken down than the more stable bodies with which I have experimented. (3) This conclusion is in harmony with the evidence of Frankland,⁵ Thatcher,⁶ Schittenhelm,⁷ and others, to the effect that certain bodies, particularly amines and urea, will give off ammonia on distillation with sodium carbonate.

Dr. Frankland, in a paper on the analysis of water, quotes experiments on the analysis of polluted waters, in which he shows that even in that case the free-ammonia determination is seriously in error, owing to the decomposition of certain substances on distillation, notably urea. Thatcher, in a thesis at the Massachusetts Institute of Technology, has also shown that amines and other nitrogenous bodies break down on distillation in alkaline solution, forming free ammonia. Schittenhelm obtained similar results in the distillation of feces. Cohn⁸ states in his work on *Reagents* that aldehydes give a brownish coloration with Nessler's reagent.

THE ALBUMINOID AMMONIA.

After the distillation of the free ammonia, as previously described, 20 c.c. of an alkaline solution of potassium permanganate is added to the sample, and the distillation continued, taking off two more tubes, or 100 c.c. The ammonia thus recovered in the distillate is read in the same way as the free

ammonia and designated "albuminoid ammonia." It is, in fact, a portion of the nitrogen of the nitrogenous matter of the sewage which is given off in the distillation as ammonia.

The albuminoid-ammonia test was first proposed by Wanklyn in 1867. It was first announced that it gave the whole of the organic nitrogen. Then in a later paper Wanklyn claimed that he obtained a constant proportion of that total, about two-thirds of it. In the following year E. Frankland⁵ showed by a series of experiments with the process that the ratio was not two-thirds, and, in fact, that it was not a constant figure at all. However, owing to its simplicity and roughly relative results, the test has been retained in the routine analysis of potable waters, and thence has been adopted without change in the analysis of sewage.

In water analysis it is claimed, and in the case of unpolluted waters it is doubtless true,⁹ that the amount of nitrogen obtained in the distillation is a constant proportion of the total, and hence the figure is of value in the interpretation of the analysis. This relation does not, however, hold even approximately with sewages and sewage effluents. Besides the nature of the substance itself, there are certain details in the process which influence the result. Experiments are here reported showing the effect of varying some of these factors.

Concentration of the permanganate in the solution.—The amount of permanganate solution added is found to have an influence upon the result. A sample of casein in solution, yielding by the regular method 8.8 parts of albuminoid ammonia per million, gave with double the amount of permanganate a value of 10.4 parts, and with one-half the correct amount a value of 4.0 parts; or, expressed in percentages, the higher concentration gave 118 per cent., and the lower 45 per cent., of the true value obtained by the standard procedure. It will be apparent that if the amount of sewage solution added varies, there will be a similar variation in the concentration of the permanganate with its consequent error. A sample of sewage treated in the same way gave similar results.

Rate of distillation.—Stress is usually laid upon this point in the text-books. A little consideration will show, however, that

the temperature will vary little, if at all, with a variation in the rate of distillation, and that, since the reaction goes on rapidly at first, and continually decreases in rate, a slower distillation is equivalent to an addition of time at the end of the distillation, when the reaction is practically completed. In short, it would appear that practically the same amount of ammonia will be yielded in a given time, whatever may be the rate. Within ordinary limits, this is found to be the case. The solution of casein used in the last experiment was also used in this one. A distillation was made at the ordinary rate of three tubes, or 150 c.c., in twenty-five minutes, and a second one at a rate of three tubes in fifty minutes. The second distillation gave a reading of 106 per cent. of the first, and the amount coming off in the first twenty-five minutes of the second case exactly equaled that obtained in the total twenty-five minutes in the first. Experiments with sewage at three different rates confirmed these results. It is therefore the *time* of distillation rather than the rate which has an influence upon the result.

Solubility of the nitrogenous substance.—It might be expected that the solubility of the substance giving up the ammonia might exert some influence upon the rate of the reaction. To test this point egg albumen was used. A solution was made of this substance, and a portion taken for analysis. A second sample was heated gently with mercuric chloride to coagulate it, and then submitted to analysis. The results obtained were 5.40 and 5.24 parts of nitrogen per million respectively. This experiment has some bearing upon the results in filtration work, and especially in the septic tank effluents; for, other things being equal, the soluble substance will give off relatively more of its nitrogen than the insoluble.

Percentage of total nitrogen recovered as albuminoid ammonia.—Having shown some of the minor variations which may be expected in a sewage analysis, I will now report some work undertaken to find out just what percentage of the total nitrogen we may expect to get off in the albuminoid-ammonia process, under the standard conditions of analysis. I will assume that the Kjeldahl process for total organic nitrogen gives that figure

with sufficient accuracy. Some doubt has recently been thrown upon the correctness of this view^{10,11} but for small quantities of nitrogen I believe the Kjeldahl results to be accurate. In some control experiments made with the Gunning modification of the Kjeldahl process and, in parallel, with a much simpler modification, to be described later, theoretical results were obtained within the accuracy of the Nessler reading, urea and asparagin being used. Next a number of substances likely to be found in sewage were analyzed by the albuminoid-ammonia process, and by the Kjeldahl, and the percentages of the total nitrogen given off in the former were calculated. The results were as follows;

Substance	Percentage of Total Nitrogen Recovered as "Albuminoid Nitrogen "
Egg albumen - - - - -	32
Vitelline - - - - -	18
Casein - - - - -	21
Gelatin - - - - -	42
Grass extracts —	
Cold water - - - - -	54
Hot water - - - - -	48
Chloroform (chlorophyll) - - - - -	29
Peptone (Witte's) - - - - -	30
Asparagin - - - - -	73
Urea - - - - -	4
Tap water - - - - -	45

Similar work on sewages gave, as an average of fifteen analyses, a value of 34.8 per cent., with maximum and minimum values of 15.2 and 54.8 per cent. These wide variations merely show the impossibility of forming any estimate of the amount of nitrogenous material present in a sewage from its albuminoid ammonia. Taking twice the albuminoid as a total, we get a maximum error of 70 per cent.; with three for the factor, the maximum error is 34 per cent.; while, with the formula proposed by Mr. Fuller, a much closer agreement is found in all cases, although the maximum difference in this case is 25 per cent. of the total. The formula includes the free ammonia as well as the albuminoid. It is

$$\text{Total nitrogen} = \frac{12x \text{ (albuminoid nitrogen)}^2}{\text{nitrogen of free ammonia}}$$

It seems that, in view of these results, and of those quoted by Fuller, the albuminoid-ammonia process, as applied to sewage, has very little value, and might safely be replaced by the Kjeldahl process, or some modification of that process.

The writer finds, by a comparative study, that the simple method used by Palmer¹² in his study of the streams polluted by Chicago's sewage, gives as good results as either the Kjeldahl or the Gunning process. Palmer's method consists merely in digesting with sulphuric acid until the liquid clears up, and then neutralizing and distilling off the ammonia formed. With sewage the method can be made very rapid, half an hour sufficing for the complete digestion. In about half the cases tried in this way it was found, by making up the digested sample to 250 c.c., and taking out 2 c.c., neutralizing and making up to 50 c.c., that the tube could be directly nesslerized. In other cases this could not be done, a turbidity occurring on nesslerizing. Lack of time has prevented a detailed study of this point, but it is believed that some way will be found of overcoming the difficulty, and that it will be possible to nesslerize the solutions directly. If this is so, it will be possible to make a determination of the free ammonia by the direct method, and of the total nitrogen by the Kjeldahl method, in about the same time that is now required for the distillations.

CONCLUSIONS.

In conclusion, I would sum up the more important points shown by this work:

All free and saline ammonia is recovered in the distillate by the ordinary process.

Many albuminoid and other nitrogenous substances decompose on boiling with sodium carbonate, giving off free ammonia, causing an error in both the free and the albuminoid ammonia values.

The percentages of the total nitrogen given off as albuminoid ammonia are so variable that no indication of the total nitrogen can be obtained from the albuminoid-ammonia figures.

A simplified Kjeldahl process and a method for the direct reading of ammonia are suggested which, together, can be made

in about the same time as the two distillations as carried out at present, and both of which are constant and accurate.

If, upon further study, the facts brought out here are found to be correct, the question arises whether the time has not come for some such official body as the Standard Methods Committee of this section to make formal inquiry, and, if they deem it wise, to make official recommendations in regard to the best and most desirable method of procedure in sewage analysis.

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A SIMPLE TEST FOR THE ROUTINE DETECTION OF THE COLON BACILLUS IN DRINKING WATER.*

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THE test which I propose for the routine detection of the colon bacillus in water depends mainly upon their organism's well-known gas-producing characteristics, which have been previously described by Theobald Smith.¹ Its influence upon the anilin dye known as neutral-red is also of importance from a diagnostic standpoint, and a brief review of the experiments with this dye is therefore in order.

Rothberger² first pointed out the difference between cultures of the colon bacillus and those of the typhoid bacillus when grown in neutral-red agar tubes. The colon bacillus produced a yellow fluorescent appearance, while the typhoid bacillus caused little or no change in the port-wine-red color of the neutral-red agar. He thought that this change was caused by a process of reduction, since exposure to the air brought back the original color.

Savage,³ Irons,⁴ and others have applied this test to the routine examination of drinking water for the presence of *Bacillus coli communis*, and Irons made a series of comparative tests between 285 specimens of waters inoculated into fermentation-tubes and into neutral-red broth cultures. The reduction of the port-wine-red color of the neutral-red broth took place in 47 per cent. of cases, while only 35 per cent. of the dextrose fermentation-tubes gave the characteristic gas formation of about 33 per cent., which is considered as an almost accurate indication of the colon bacillus. Irons again examined all of the specimens which reduced neutral-red broth, but which failed to produce 33 per cent. gas formation in dextrose bouillon in the fermentation-tube.

*Read at the meeting of the Laboratory Section of the American Public Health Association, October 26, 1903.

He found that in every case except three he could isolate other bacteria which reduced neutral-red, while he failed to demonstrate the presence of the colon bacillus in all but two of these twenty-two specimens. It can be granted, therefore, that Whipple's dextrose fermentation-tube test furnishes a more accurate test than the neutral-red bouillon tubes for the routine detection of *Bacillus coli*, since in addition Irons isolated various other bacteria from river water belonging to the enteritidis, proteus, cloacae, and non-gas-producing groups which reduced neutral-red in the bouillon test-tube.

Gage and Phelps⁵ have used neutral-red in bouillon and shake-agar cultures in the routine detection of *Bacillus coli communis*, but, upon finding that a large number of other bacterial species caused the same reduction of red to yellow, they concluded that it was of little value as a confirmatory test of the colon bacillus.

Jordan,⁶ in his investigation of the river waters in Illinois, has made an accurate classification of the various types of fermentative bacteria found in water, and this work has greatly helped the search for a simple test for isolating the colon bacillus from water.

Groups I and II, which gave the characteristic cultural and fermentative reactions of *Bacillus coli* and *Bacillus lactis aërogenes*, can be passed without further comment, but Groups III and IV, with their subdivisions, need further mention.

Group III liquefies gelatin, casein, and blood serum, and ferments glucose and sucrose, but not lactose, Subdivision 1 forming less than 30 per cent. of gas in the fermentation-tube, Subdivision 2 not liquefying gelatin, while Subdivision 3, called the cloacae group, produces over 50 per cent. of gas in glucose and sucrose bouillon. Jordan mentions the fact that a few of these types in Subdivision 3 slowly ferment lactose; but it will be admitted by all, I think, that even this type will not produce 30 per cent. of gas in forty-eight hours. As I shall later lay great stress upon the specific action of the colon bacillus upon lactose bouillon, this point has been mentioned at this time. Group IV resembles mainly the colon bacillus, but alkalizes milk, and only produces gas in dextrose bouillon, not affecting lactose or sucrose. Its failure to ferment lactose is thus of diagnostic importance.

TABLE I.

GROUP I—B. VULGARIS.						GROUP VI — B. ENTERITIDIS (HOG-CHOLERA GROUP).					
					Gas Prod. and Ratio.						Gas. Prod. and Ratio
Glucose	-	-	-	-	20%	Glucose	-	-	-	-	40-50%
					Carbon-dioxide, 1 part						Carbon-dioxide, 1 part
					Hydrogen, 1 part						Hydrogen, 2 parts
Lactose	-	-	-	-	6-10%	Lactose	-	-	-	-	0
Sucrose	-	-	-	-	25%	Sucrose	-	-	-	-	0
					Carbon-dioxide, 1 part						
					Hydrogen, 2 parts						
 GROUP II — B. CLOACAE.						 GROUP VII — B. PRODIGIOSUS.					
Glucose	-	-	-	-	60-100%	Glucose	-	-	-	-	40%
					Carbon-dioxide, 2 parts						Carbon-dioxide, 1 part
					Hydrogen, 1 part						Hydrogen, 2 parts
Lactose	-	-	-	-	0-10%	Lactose	-	-	-	-	90%
Sucrose	-	-	-	-	60-100%						Carbon-dioxide, 2 parts
					Carbon-dioxide, 2 parts						Hydrogen, 1 part
					Hydrogen, 1 part	Sucrose	-	-	-	-	80%
											Carbon-dioxide, 2 parts
											Hydrogen, 1 part
 GROUP III — B. CLOACAE (NON-LIQUEFACIENS).						 GROUP VIII — B. PYOCYANEUS.					
Fermentation formula same as Group II.						No fermentation of sugar bouillons.					
 GROUP IV — B. GASOFORMANS.						 GROUP IX — B. PSEUDO-COLI.					
Glucose	-	-	-	-	80%	Glucose	-	-	-	-	85%
					Carbon-dioxide, 2 parts						Carbon-dioxide, 1 part
					Hydrogen, 1 part						Hydrogen, 2 parts
Lactose	-	-	-	-	90-100%	Lactose	-	-	-	-	40-70%
					Carbon-dioxide, 2 parts						Carbon-dioxide, 2 parts
					Hydrogen, 1 part						Hydrogen, 1 part
Sucrose	-	-	-	-	90%	Sucrose	-	-	-	-	90%
					Carbon-dioxide, 2 parts						Carbon-dioxide, 2 parts
					Hydrogen, 1 part						Hydrogen, 1 part
 GROUP V — B. GASOFORMANS (NON-LIQUEFACIENS).						 GROUP X — B. COLI.					
Fermentation formula same as Group IV.						Glucose	-	-	-	-	40-50%
											Carbon-dioxide, 1 part
											Hydrogen, 2 parts
						Lactose	-	-	-	-	Same
						Sucrose	-	-	-	-	0-30%
											Carbon-dioxide, 1 part
											Hydrogen, 2 parts

NOTE.—This classification is based upon the findings of the author and the groups described by Jordan. Fuller and Johnson describe a *B. pyocyaneus* that ferments glucose, but the recent work with this organism seems to indicate that the true *B. pyocyaneus* does not ferment the various sugars with gas production. It is not claimed that these figures and ratios will not vary, but the present table is simply meant to form the basis for a working hypothesis for further work.

TABLE II.

		Neutral-Red Reaction in Lactose	50% of Gas in Lactose. CO ₂ -1-H-2
Group I	<i>B. vulgaris</i>	Positive	Negative
Group II	<i>B. cloacae</i>	Negative	Negative
Group III	<i>B. cloacae</i> (non-liquefaciens)	Negative	Negative
Group IV	<i>B. gasoformans</i>	Negative	Negative
Group V	<i>B. gasoformans</i>	Negative	Negative
Group VI	<i>B. enteritidis</i>	Positive	Negative
Group VII	<i>B. prodigiosus</i>	Negative	Negative
Group VIII	<i>B. pyocyaneus</i>	Negative	Negative
Group IX	<i>B. pseudo-coli</i>	Negative	Negative*
Group X	<i>B. coli</i>	Positive	Positive

Group I corresponded to the type of *Proteus vulgaris*, or *Bacillus vulgaris*, and was isolated in fifty-eight instances. These organisms produced about 20 per cent. of gas in glucose and sucrose bouillon, but lactose bouillon showed no gas formation. Twenty specimens did not liquefy gelatin. Group II was similar to the subdivision of Jordan's proteus group which he called the cloacae subdivision. These bacteria produced from 60 to 100 per cent. of gas in dextrose (glucose) and sucrose, but lactose never showed more than 10 per cent. of gas. Gelatin, serum, and casein were rapidly liquefied. Group III gave the same fermentation formula as Group II, but neither gelatin, serum, nor casein was liquefied. This I called *Bacillus cloacae* (non-liquefaciens). Forty-four specimens of Group II and eighty-six of Group III were isolated from the various waters.

Group IV has been included in the proteus group by Jordan, but I think that this group deserves a special classification. This has been named *Bacillus gasoformans* because it produces rapid gas formation in dextrose, lactose, and sucrose. Dextrose shows about 80 per cent., lactose 100 per cent., and sucrose 90 per cent.

*Positive once in 567 specimens.

of gas in the fermentation-tube. This organism liquefied gelatin, and was isolated twenty-two times.

Group V resembled Group IV, but did not liquefy gelatin. It was called *Bacillus gasoformans* (non-liquefaciens), and was obtained in thirty-three instances.

Group VI corresponded to *Bacillus enteritidis* and produced about 40 per cent. of gas in dextrose bouillon, but did not affect lactose and sucrose. Thirteen specimens of this group were obtained, of which seven rendered milk alkaline, and six acidulated milk. One specimen liquefied gelatin.

Group VII was *Bacillus prodigiosus*, which formed a brilliant red pigment and liquefied gelatin rapidly. It produced 50 per cent. of gas in glucose, about 90 per cent. in lactose, and about 70 per cent. in saccharose bouillon.

Group X was the *Bacillus coli communis*, and its well-known property of producing about 50 per cent. of gas in dextrose and lactose need hardly be mentioned.

Bacillus coli was isolated 295 times, and fifteen specimens were obtained from water which corresponded to *Bacillus coli* in every particular, excepting that they liquefied gelatin, and usually produced from 60 to 100 per cent. of gas in sucrose bouillon.

In all, 567 fermentative bacteria was isolated from the various water supplies throughout the state of Maryland and the city of Baltimore.

I have described the various types of gas production, in order to point out the fact that the colon bacillus produces results in the lactose bouillon fermentation-tube which might be considered as characteristic. The members of the other groups mentioned above either produce more or less gas than the *Bacillus coli* in lactose bouillon, and when more gas is produced the ratio of CO_2 to H is reversed as compared to that of the colon bacillus.

While using the usual 1 per cent. solution of 0.5 per cent. aqueous neutral-red in bouillon, it occurred to me that this same method might be applied to fermentation tubes, and I added 0.1 gr. per liter of neutral-red to all of the three sugar bouillons in the fermentation-tubes.

The routine use of this dye has proved that the colon bacillus

apparently always produces a typical color reaction in all three tubes, although some other organisms produce the same result.

In dextrose and lactose the reaction is especially marked, and consists in a reduction of the port-wine-red color of the bouillon in the closed end of the fermentation tube to a clear canary-yellow, or at times a darker orange color. The open bulb remains red, and a sharp line of demarkation between the yellow and red color takes place in the stem uniting the open and closed bulbs.

After carefully examining the fermentation formulas of 567 gas-producing bacteria, I have been able to find only sixteen specimens which produced gas varying from 30 to 60 per cent. in lactose bouillon. These were called Group IX, or *B. pseudo-coli*.

These were all members of *Bacillus gasoformans* group; and if we relied entirely upon the 50 per cent. of gas production in lactose bouillon, we should have a percentage of error of 2. All but six of these organisms gave an inverted gas formula, however; that is, 2 parts of CO_2 to 1 part of H_2 ; and although I have tested only four in lactose neutral-red bouillon in the fermentation-tube, in every case but one the entire medium was turned yellow, and did not show the typical contrast of port-wine-red and yellow, so typical of *Bacillus coli*. I have also inoculated neutral-red glucose fermentation-tubes with *Bacillus coli* in combination with one of each of the above groups, and in every instance the colon bacillus maintained its typical gas production, and produced the contrast—yellow-red reaction.

I have examined 567 gas formulas, and have found only six of these corresponding to the formula in lactose bouillon of *Bacillus coli* which failed to give the other tests for the colon bacillus. Three of these turned neutral-red entirely yellow in both branches of the fermentation-tube, leaving one in 567 specimens (two not tested in lactose) which could have been mistaken for the colon bacillus, if direct inoculation of fermentation-tubes had been employed, without further plating and isolation.

Neutral-red lactose bouillon gave the yellow-red contrast reaction with *Bacillus proteus*, *Bacillus Salmonii*, *Bacillus cloacae*, and *Bacillus enteritidis*; but, of course, no gas was produced; while many specimens of *Bacillus coli* gave the typical yellow-red

color reaction as well as the gas production. The *Bacillus prodigiosus* turns lactose entirely yellow and produces 90 per cent. of gas in lactose. I have not had the chance to apply the other members of Jordan's chromogenic group to the lactose neutral-red test, but these are the only gas-producing water bacteria which I have not used, as far as I know, since it seems to be well established that *Bacillus pyocyaneus* does not produce gas, and this accords with my own experience.

In conclusion, I think that the combination of the production of from 30 to 50 per cent. of gas, the proportion of 1 part of carbon dioxide to 2 of hydrogen, and the neutral-red yellow-red contrast reaction in lactose bouillon in the fermentation tube forms a characteristic reaction for *Bacillus coli*.

I therefore propose it as a provisional test for the present, always to be controlled by isolation in pure culture. I hope that further work will prove that the isolation in pure culture is unnecessary. We shall then be able to substitute the simple inoculation of a fermentation-tube with varying amounts of water for the wearisome week's work now in vogue for the isolation of the colon bacillus from water.

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ISOLATION OF BACILLUS COLI COMMUNIS FROM THE ALIMENTARY TRACT OF FISH AND THE SIGNIFICANCE THEREOF.*

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IN the selection of a public water supply it often happens that a decision regarding the sanitary character of the same is based almost wholly upon analytical evidence. For want of a better substitute, the colon bacillus has been quite generally accepted as an index to pollution: and although opinion varies widely regarding the absolute value of the colon test in sanitary water analysis, and although it is to be admitted that it has its limitations, yet nearly everyone, including the most doubtful, is inclined to look with suspicion upon a water, which, on analysis, discloses the presence of the colon bacillus.

More has been written in recent years regarding this organism and allied forms than upon almost any other topic relating to the bacterial analysis of water. The value of the qualitative test for *B. coli communis* has been questioned for three reasons; namely, (1) because the presence of a few of these organisms in a water does not necessarily mean that it is made unfit for primary purposes thereby; (2) because there are numerous saprophytic forms which show, upon study, cultural characteristics identical, or nearly identical, with those shown by the colon bacillus; and (3) because the fact that the colon bacillus is occasionally found in apparently pure waters does not necessarily imply that the typhoid bacillus is also present.

Of the more recent writers on the subject Prescott¹ has pointed out that bacteria corresponding in every way to *B. coli* are not confined to animal intestines, but are widely distributed elsewhere in nature. He says: "Certain lactic-acid bacteria isolated from sources apparently free from contamination are absolutely identical with the colon bacillus." Moore,² on the other hand, states

* Read at the meeting of the Laboratory Section of the American Public Health Association, October 26, 1903.

that, no matter if the colon bacillus is found elsewhere than in the digestive tract of living animals, including man, to his knowledge it has never been satisfactorily demonstrated in any substance where the possibility of tracing it to this source was precluded. The demonstration of this organism in sources other than the digestive tract, in sewage and in water, does not appear to the writer to depreciate the value of the colon test in water analysis as a perhaps presumptive, but valuable, index to fecal contamination.

Other cases than those cited above are constantly noted where opinion regarding the value of the colon test is divided. Be this as it may, it is still the case that, even in the face of adverse criticism, the presence of *B. coli communis* in water is widely considered as an unfavorable indication, and the sanitary character of such water suffers materially in consequence.

It is, of course, well known that the reason for such wide recognition of the value of this organism as an index to fecal pollution lies in the fact that it is essentially a germ of sewage origin. Some writers have even gone so far as to claim that *B. coli communis* and *B. typhosus* are one and the same, possessing divergent cultural characteristics at times, but under favorable conditions capable of exerting a similar action upon man in bringing about enteric disorders.

It is not the purpose of this paper to attempt to unravel the badly knotted skein of evidence for or against these theories. It is enough to assume that *B. coli communis* has its specific value in indicating the impurity of a water supply. It is enough to be assured that this organism and *B. typhosus* are found together, even though it is known that, while the former is always present where the latter is found, the latter is not always found to exist in common with the former. It is an indisputable fact that *B. coli communis* is a constant inhabitant of intestinal matter. It is equally certain that it is not a constant inhabitant of sources other than this.

It is the opinion of the writer that, if the value of the colon test in sanitary water analysis is depreciating, it is not because it lacks substantial ground as a basis for its claims

as an index to fecal contamination, but because laboratory methods for its study are not improving along systematic lines with the rapidity or certainty that are imperative. As Moore² has very aptly pointed out, a closer knowledge of the but indifferently understood forms connecting the accepted colon bacillus and its prototype is required. It is true that there are many forms of bacteria which closely resemble *B. coli*, but the fact they fail in some specific test does not prove, in the opinion of the writer, that they are not *B. coli*. It is equally true that the colon bacillus at times will reach a stage of deterioration where it will not respond to one-half the prescribed tests. But it is the belief of the writer, that if preliminary cultivation is resorted to before final results of growth are recorded, a much closer adherence to fixed characteristics will be noted in the study of the colon bacillus. A more thorough preliminary cultivation than was suggested some three years ago³ might not be unwise, and it would appear that, by taking advantage of this point, bacteriologists may not only be able to bring together the numerous varieties of the colon bacillus under one head, but also always to obtain constant results in a study of this organism in water.

ISOLATION OF BACILLUS COLI COMMUNIS FROM FISH.

There appear to the writer but three ways in which the colon bacillus may become the inhabitant of a water; namely: through the introduction of sewage, through the direct agency of man and animals, and through the agency of fish.

Some time ago the writer arrived at the decision that fish might well be admirable vehicles for the transportation of bacterial life from one body of water to another. It appeared perfectly feasible to him that a fish in its passage from a polluted into a comparatively pure water might carry with it the colon bacillus which, if subsequently isolated therefrom, would entitle the water under study, in accordance with existing ideas, to a place among suspicious, even if not polluted, waters, and unfit for domestic consumption.

So far as is known to the writer, there is no published literature bearing directly on this subject, although I am informed by

Mr. G. C. Whipple that something in this line was done by him some years ago. In Mr. Whipple's study of the intestinal contents of some fifty or seventy-five fresh-water fish he was unable to find the colon bacillus. It is worthy of note, however, that the fish studied by him—including trout, white perch, and sunfish—were caught in an obviously unpolluted water.

Occasion offered itself for a study of the point in question at St. Louis, Mo., during the year of 1900, and the results of this work appear to the writer to be of sufficient interest to be placed on record. Acknowledgment here is due to the assistance of Mr. C. A. Snodgras, who in a painstaking manner worked out the results given below. The writer wishes also to express his indebtedness to Dr. Amand Ravold for many valuable suggestions during the course of the work.

In preparing for this work, arrangements were made whereby freshly caught fish of well-known species were expressed to the laboratory, and immediately upon their arrival were opened, and tubes of broth* inoculated with the contents of the large intestine and stomach of each.

From these cultures, after twenty-four hours' incubation at body temperature, lactose litmus agar plates were prepared, three plates to each tube. These plate cultures were incubated for from twenty-four to forty-eight hours at body temperature, and characteristic colonies transferred therefrom to fermentation-tubes containing dextrose broth. These cultures were allowed forty-eight hours' incubation at 37° C., and at the expiration of that time, if gas was absent from any of the tubes, further study in such cases was discontinued. Work in the case of the tubes showing gas production was continued as follows:

Lactose litmus agar plates were prepared, three plates from each fermentation-tube, and incubated for from twenty-four to forty-eight hours at body temperature. Characteristic colonies were selected from these plates, and usually two or three stock cultures were prepared on slant agar in the case of each plate. When the stock cultures had developed sufficiently, the following

*Liebig's beef extract, 10 g.; peptone, 10 g.; dextrose, 10 g.; Parietti solution, 20 c.c.; distilled water, 1,000 c.c. Reaction before addition of Parietti solution, neutral to phenolphthalein.

media were inoculated therefrom: nutrient broth tube, gelatin tube, fermentation tube, peptone solution for indol, nitrate solution, and litmus milk.

The cultures giving the following reactions were judged to be *B. coli communis*:

Bacillus, true form, obtained in three-day agar cultures.

Motility in two-day broth cultures.

Broth rendered turbid.

Gelatin tube, luxuriant growth along the entire path of the needle, without liquefaction of the gelatin.

Fermentation of dextrose broth with a total gas production of from 25 to 75 per cent. Ratio of H to CO₂ approximately 2 : 1.

RESULTS OF TESTS FOR *BACILLUS COLI COMMUNIS* IN THE ALIMENTARY TRACT OF FISH.

Fish	Where Caught	Number Examined	NUMBER IN WHICH <i>B. COLI COMMUNIS</i> WAS FOUND	
			Intestine	Stomach
German carp	Illinois River	13	9	8
German carp	Mississippi River	3	..	1
Buffalo	Illinois River	4	4	3
Buffalo	Mississippi River	5	5	3
Spoonbill cat	Mississippi River	1	1	..
Silver carp	Illinois River	2	1	..
Silver carp	Mississippi River	4	4	1
Gar	Illinois River	1
White perch	Illinois River	7	2	2
White perch	Mississippi River	4	2	1
Black bass	Illinois River	1
Cat	Illinois River	7	5	1
Cat	Mississippi River	2	2	1
Sunfish	Mississippi River	1	1	..
Eel	Illinois River	1	1	..
Eel	Mississippi River	1
Croppie	Mississippi River	1
Sturgeon	Illinois River	1	1	1
Sturgeon	Mississippi River	2	..	1
Tooth herring	Mississippi River	2	1	1
Sucker	Mississippi River	2	1	..
Dogfish	Illinois River	1	1	..
Dogfish	Mississippi River	1

Total number of fish examined - - - - - 67

Total number of fish from which *B. coli communis* was isolated - - 47

Number of fish in the stomach of which *B. coli communis* was found - 24

Number of fish in the intestines of which *B. coli* was found - - 41

Indol produced.

Nitrate reduced to nitrite.

Milk coagulated without liquefaction of the casein; litmus reddened.

Fecal odor of growth in broth and agar cultures, respectively.

All of the above tests, except where specifically stated, were made after ten days' incubation at body temperature. The results of these studies are given above.

MULTIPLICATION OF *B. COLI COMMUNIS* IN THE INTESTINAL TRACT OF FISH.

Further work was done to determine whether this organism multiplied to any extent in the intestinal tract. Infusions were prepared by suspending the intestinal contents of several fish in distilled water. These infusions, after sterilization, were inoculated with the colon bacillus. Having in mind the fact that cooked organic matter offers better opportunity for the nourishment of bacterial life than otherwise, infusions were also prepared by suspending the intestinal contents in sterilized distilled water and filtering the same through a Berkefeld filter, instead of resorting to sterilization as in the case of the other infusions. These latter infusions, while not found to be absolutely sterile in all cases, were practically so. They were also inoculated with the colon bacillus.

The results of this experiment showed a very pronounced increase in numbers of *B. coli* after three days' standing at a temperature of 20° C., not only in the infusions sterilized before inoculation, but in the filtered infusions as well. Original plates made from the inoculated infusions showed from 100 to 1,000 coli per cubic centimeter, while after three days the number had increased to many hundreds of thousands.

CONCLUSIONS.

It has been shown that the colon bacillus is taken up by fish, and the results of the experiment described above indicate that it multiplies rapidly in the intestinal tract of the same. It has been shown that a search of a large number of fish caught in an unpol-

luted water failed to reveal the presence of this organism in the intestinal tract. It would seem possible, therefore, that fish, having taken up the colon bacillus from a polluted water might migrate to a water of comparative purity, where they would naturally discharge the greatly increased number of these organisms.

Whether the finding of the colon bacillus deposited in a water in this manner would depreciate the value of the colon test in the examination of a public water supply is a question. It would certainly appear that, if the colon bacillus can be thus easily transferred from one water to another, the transportation of the typhoid bacillus may be considered quite as likely. At least the above results may explain the apparent phenomenon frequently noted where *B. coli communis* is found in comparatively large numbers in waters apparently open to but remote chances for fecal contamination.

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SODIUM SULPHITE: A DANGEROUS FOOD-PRESERVATIVE.*

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THE extent to which sodium sulphite is used as an admixture to meat and canned vegetables is not commonly known, and those who are aware of its use are generally misinformed as to the reason of its employment. It is classed as a food preservative, but its antiseptic properties are comparatively feeble. It is used more especially on account of its effect on the appearance of the food to which it is added, its preservative influence being decidedly a minor consideration. It confers upon mince meat an abnormally brilliant red color, which conveys to the purchaser the idea of freshness; and it has a bleaching effect on canned asparagus and canned corn, thus making these articles more attractive to the eye. Its most extensive use is in the preparation of that form of minced meat which we know as "Hamburg steak." This is made from beef trimmings and inferior parts of the carcass; and after it has received its chemical treatment, it takes on a redness that is much more pleasing to the eye than the grayish-brown color which develops within a few hours if no sulphite is added. This color, due to the fact that the salt favors the formation of oxyhemoglobin, is very persistent on the exterior of the mass, but is not so marked in the inner portions, which, however, acquire the same appearance on exposure to the air. Persisting as it does, meat which is in reality well advanced in decomposition is readily disposed of as perfectly fresh, for although the number of bacteria per gram may run as high as 500 millions, it may give off no marked odor. Aside, then, from the effects of the compound on the system, it may fairly be said that, on account of its masking decomposition, it is an undesirable admixture.

The first experiments which yielded definite results showing that the salt acts poisonously on the system were reported in 1896

* Read at the meeting of the Laboratory Section of the American Public Health Association, October 26, 1903.

by Kionka,¹ who, working with dogs, discovered evidence of extensive injury in practically all of the organs. His results were accepted for a time as conclusive; but, in 1901, Abel² reported that daily doses of 0.5 to 1.0 gram, and once as much as 2.5 grams, taken by himself and seventeen others for twelve days, caused no discomfort nor observable effects of any kind whatever. Then, Lebbin and Kallmann³ fed young dogs for sixty days with meat containing 0.20 per cent., and found no lesions on post-mortem examination. They gave large doses (10 grams) in 25 per cent. and 40 per cent. solutions to rabbits by means of the stomach-tube, and observed nothing more serious than diarrhea and slight congestion of a portion of the gastric mucosa. An equal weight of common salt in 30 c.c. of water caused death within two hours. Finding, in addition, that three men, who, for three days, ate meat containing 0.10 per cent. of sulphite, were not made sick, they assailed Kionka's work, and, with others, contended that sulphites should be regarded as harmless in all respects. This led Kionka⁴ to repeat his experiments with a larger number (six) of dogs, which he fed nine weeks with meat containing 0.20 per cent., which is the proportion commonly recommended. During the whole period, none of the animals showed any outward sign of poisoning; and, in fact, all gained in weight up to the day they were killed, when post-mortem examination showed in each subject evidence of marked degenerative changes in various organs. Numerous small hemorrhages were observed in the lungs, endocardium, stomach, intestines, and liver, and all showed acute nephritis. Practically the same results were obtained, also in dogs, by Schulz.⁵ Kionka repeated Lebbin's experiments with rabbits, and found that they died very quickly.

A number of writers have reported discomfort, eructations, headache, and other effects attributed to treated meats, and others have recorded negative results from similar experience, but, for obvious reasons, such evidence is hardly worthy of discussion.

Before I happened to see Kionka's second paper and Schulz's communication, I began an experiment with cats, giving them a daily feed of freshly hashed beef, to which was added 0.20 per cent, of pure crystallized sodium sulphite. While it was in progress I,

saw both papers and concluded to extend the time considerably beyond that covered by Kionka's experiment. Six cats were under observation, one being a control and receiving the same weight of untreated meat. From the start, all six of the animals, which were of the homeless, ill-fed, scavenging kind, gained in weight, but about the ninth week, at which time Kionka killed his dogs, all except the control began to lose. The weekly losses were not constant: sometimes slight gains were observed; but these were succeeded by larger losses, so that the general trend of the weight-curve was downward. At no time did any of the animals show any outward evidence of poisoning up to five months, when all were killed. On section, the macroscopic appearances were negative in all respects: but microscopic examination of the hardened organs, made by Dr. Tyzzer, of the Pathological Laboratory of the Harvard Medical School, showed, in every subject, excepting the control, a parenchymatous degeneration of the kidneys. Each showed cloudy swelling and marked fatty degeneration of the renal epithelium; and in one case there was an acute interstitial nephritis in addition to and probably dependent upon parenchymatous changes. Otherwise, the results of the examination were essentially negative. The control cat showed no pathological changes whatever. She continued to gain in weight to the end of the experiment.

Although the lesions produced were much less extensive than those observed in Kionka's and Schulz's dogs, we may conclude that, at least for animals, and probably for the human subject, sodium sulphite is, as Kionka first stated, a dangerous admixture to food.

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STUDIES OF MEDIA FOR THE QUANTITATIVE ESTIMATION OF BACTERIA IN WATER AND SEWAGE.*

(SECOND PAPER.†)

STEPHEN DE M. GAGE AND GEORGE O. ADAMS.

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IN a report from this laboratory at the Buffalo meeting the results of studies of culture media were presented, which showed that the media in general use for quantitative work enabled us to enumerate only a small percentage of the total number of bacteria in a given volume of ordinary water, and that the small percentage so obtained was not constant, but varied with different classes of water, different periods of incubation, etc. Furthermore, it was shown that higher counts were obtained by a simplification of the usual media, and that a new medium, Nährstoff agar, gave us counts many times greater than any other. The studies reported at that time were incomplete, and although much has been accomplished in the two years which have elapsed since that paper was prepared, the studies are apparently as far from completion as before. Nevertheless, certain facts have been ascertained which have an important bearing on the composition and preparation of the media, and it is thought wise to present these for discussion at this time.

The opinion has frequently been advanced that it is not good policy to destroy the existing, until the material is at hand to rebuild from the ruins a structure of greater stability. While this is perhaps true in a general sense, in scientific work it is frequently possible to pick flaws and determine errors in existing methods; and while data of this class have the tendency to destroy somewhat the confidence of the uninitiated in these methods, an impetus is given to research work which will hasten the rebuilding.

* Read at the meeting of the Laboratory Section of the American Public Health Association, October 25, 1903.

† The first paper, under the same title, by GAGE AND PHELPS, appeared in *Jour. Am. Pub. Health Assn.*, 1901, 27, p. 333. Mr. Phelps was succeeded by Mr. Adams in January, 1903.

The data here presented are of this class—partly destructive of the present system, and at the same time serving as a ground-work for improved and more accurate methods for quantitative bacterial analyses.

A number of papers have recently appeared bearing on the different phases of the media question, but it is not the purpose of the writers to enter into a review of the literature of the subject. Two papers, however, may be mentioned in passing. Whipple* furnishes data which prove conclusively that we can never hope for any degree of accuracy or uniformity in quantitative bacteriology until we relegate gelatin media to the place to which they belong—that of confirmatory media for qualitative work or special investigations. Gustav Hesse† presents accurate data on the effect of neutralization with various alkalies and acids, on the change in reaction during sterilization, and on the effect of combinations of these two problems on the numbers of bacteria as determined by different media.

METHOD OF EXPRESSING RESULTS.

Throughout, the results of counts of bacteria are expressed as relative numbers of bacteria, these being obtained by calling the actual number on some one of the media 100, and expressing the other counts as percentages of that number. By this method of expression we have been enabled to average results obtained with waters containing widely differing numbers of bacteria, without giving undue weight to those samples which contained excessively large numbers. In nearly every case the figures given are an average of a number of separate comparisons. It is often the case that colonies on a medium become obscured, and that a lower count is obtained after the maximum is reached. In the tables we have in every case carried out this maximum as the number which would be found on each of the succeeding days, in order to eliminate, as far as possible, the error due to liquefaction or spreaders.

* *Tech. Quart.*, 1902, 15, p. 127.

† "Beiträge zur Herstellung von Nährböden und zur Bakterienzüchtung," *Ztschr. f. Hyg.*, 1903, 54, p. 1.

COMPOSITION AND PREPARATION OF MEDIA.

The standard gelatin has been of the composition and method of preparation recommended by the Bacteriological Committee. The Lawrence agar is very similar to the standard agar, but contains only 1 per cent. of agar, and varies slightly in the method of preparation. All other media, the preparation of which is not individually described, are the same as those described in the former paper.

VARIATION IN THE COMPOSITION OF BEEF-INFUSION A SOURCE OF ERROR IN CULTURE MEDIA.

In the earlier days of bacteriology it was believed that some very rich medium was necessary in order to obtain good bacterial development, and various experimenters tried different infusions, finally settling on beef as the ingredient best adapted for the purpose. The use of beef in culture media is a custom so strongly ingrained in the minds of bacteriologists at the present day that it is extremely difficult to shake their faith in this ingredient. It has often been pointed out that beef-infusion and the commercial beef extract are very variable in composition, but studies covering this variation have hitherto presented only very meager data. It has always been the custom at the Experiment Station to record the reaction of the raw beef-infusion, and of the same infusion after the albumens had been coagulated by heat and filtered out.

During 1902 determinations of solids were made on every lot of beef-infusion, both before and after the albumens were removed. Of course, this is only a very rough determination of the variation in the nutrient value of the different infusions, but, taken together with the acidity, it gives us an approximate measure of this variation. In all, fifteen lots of beef-infusion were so determined. In the raw beef-infusion we found a variation both in total and organic solids of over 2 per cent. by weight of the whole infusion. After the albumens were removed by coagulation and filtration, we found a variation of nearly 1 per cent. in these organic solids, and there was a variation in the reaction of the infusion of 1.3 per cent.

In other words, we have been attempting to make a medium

of approximately uniform composition, taking great pains that all the processes shall go on under precisely the same conditions, and that the final reaction shall be minutely adjusted to a fixed point; and we are using as a basis for that medium a substance in which the natural variation in the nutrient material is greater than the total amount of accurately determined nutrients (1 per cent. pepton) which we incorporate with it. This is, of course, assuming that the organic solids, as determined by the loss on ignition, are an approximate measure of the material in the infusion. The results of the analyses of the different lots of beef-infusion are shown in Table I:

TABLE I.

Variation in Total and Organic Solids and in Reaction of Different Lots of Beef-Infusion.

BEEF- INFUSION No.	SOLIDS (Parts per 100,000)						REACTION	
	Raw			Coagulated and Filtered			Raw	Coagu- lated and Filtered
	Total	Organic	Fixed	Total	Organic	Fixed		
1	1,816	1,585	231	941	727	214	2.00	1.70
2	1,985	1,750	235	1,701	1,347	354	2.10	1.90
3	1,188	1,006	182	1,512	1,181	331	2.50	2.10
4	1,272	1,092	180	1,154	912	242	2.30	2.10
5	1,408	1,210	198	1,026	809	217	2.05	1.70
6	2,949	2,562	387	1,184	910	274	1.90	1.65
7	2,606	2,305	301	1,325	1,036	289	2.70	2.60
8	2,672	2,340	332	1,047	801	246	2.60	1.70
9	3,340	2,663	677	1,502	1,218	284	3.00	2.70
10	2,891	2,544	347	1,814	1,432	382	2.00	1.90
11	2,384	2,034	350	1,317	974	343	2.40	2.90
12	2,590	2,204	386	1,913	1,514	399	2.30	2.40
13	3,472	3,026	446	1,516	1,158	358	3.00	2.80
14	3,590	3,200	390	1,094	844	250	3.00	2.70
15	2,108	1,836	272	1,103	826	277	1.90	1.90
Average ..	2,418	2,091	327	1,343	1,046	297	2.38	2.18
Maximum ..	3,590	3,200	677	1,913	1,514	399	3.00	2.90
Minimum ..	1,188	1,006	180	941	727	214	1.90	1.65

COMPARATIVE VALUE OF MERCK'S AND WITTE'S PEPTON FOR
QUANTITATIVE CULTURE MEDIA.

Considerable confusion has arisen in the past through the use of various brands of pepton by different observers, and until the methods of the Bacteriological Committee recommending Witte's pepton were adopted, Merck's pepton was the one used at

Lawrence. In order to determine approximately the relative value of these two peptons, lots of pepton agar were made with each, and samples of different classes of water were plated on them, counts being made daily until the maximum count was reached, incubation being at 20° C. With four out of the five classes of water, Witte's pepton gave the higher count, and a maximum count at an earlier date than with Merck's pepton. With lake water, Merck's pepton gave better results, but these results would possibly have been changed had a larger range of surface waters been covered by the investigation, the samples being confined in this case to one source. The results of these studies are shown in Table II:

TABLE II.
Relative Numbers of Bacteria on Pepton Agar with Different Peptons.

Class of Water	Pepton	2 Days	4 Days	6 Days	8 Days	10 Days	12 Days
Sewage.....	Merck's	14	27	31	39	55	68
	Witte's	51	67	100	100	100	100
Filtered sewage.....	Merck's	4	45	65	69	69	69
	Witte's	53	69	100	100	100	100
Merrimack River ...	Merck's	16	49	62	85	93	93
	Witte's	22	39	100	100	100	100
Filtered water	Merck's	5	11	33	51	74	95
	Witte's	4	22	100	100	100	100
Lake water.....	Merck's	0	10	29	48	94	100
	Witte's	35	35	35	35	35	35
All waters	Merck's	9	33	51	67	89	98
	Witte's	38	53	100	100	100	100

Pepton agar = 1 per cent. agar, 1 per cent. pepton in water.

EFFECT OF THE KIND OF WATER USED IN MEDIA ON BACTERIAL DEVELOPMENT.

It has previously been shown that the composition of the water used in making a culture medium had considerable influence on the number of bacteria developing on the medium, and that with media made from a given water a maximum count was obtained with the same class of water. In order to study this point, five lots of plain agar were made with five different waters, as follows: regular Lawrence sewage from which the gross solids had been removed by filtration through paper; filtered sewage, *i. e.*, the

effluent from one of the sewage filters at the Experiment Station, which was giving almost complete purification; Merrimack River

TABLE III.

Chemical Analysis of Waters Used in Making Plain Agars.

(Parts per 100,000.)

Class of Water	Free Ammonia	Albu- minoid Ammonia	NITROGEN As		Oxygen Consumed
			Nitrates	Nitrites	
Sewage.....	4.1000	0.3600	0.000	0.0000	5.20
Filtered sewage.....	0.1900	0.0240	4.620	0.0028	0.28
Merrimack River.....	0.0086	0.0164	0.011	0.0002	0.45
Filtered water.....	0.0006	0.0056	0.040	0.0000	0.20
Distilled water.....	0.0088	0.0000	0.000	0.0000	0.00

TABLE IV.

Relative Numbers of Bacteria with Different Classes of Waters on Plain Agar Made with Those Waters

Water Plated	2 Days	4 Days	6 Days	8 Days	10 Days	12 Days
Plain agar made with sewage:						
Sewage	2	40	50	87	100	100
Filtered sewage	0	0	0	0	0	0
Merrimack River.....	0	11	32	32	47	47
Filtered water.....	0	0	0	0	0	0
Plain agar made with filtered sewage:						
Sewage	7	59	59	81	81	81
Filtered sewage	0	5	5	13	16	18
Merrimack River.....	26	32	42	42	42	42
Filtered water.....	21	21	21	21	21	21
Plain agar made with Merrimack River water:						
Sewage	11	46	46	64	64	64
Filtered sewage	1	1	10	18	20	21
Merrimack River.....	0	21	21	47	47	47
Filtered water.....	0	7	14	43	57	64
Plain agar made with city water:						
Sewage	15	59	64	65	76	76
Filtered sewage	0	2	23	31	41	48
Merrimack River.....	16	63	74	74	74	100
Filtered water.....	0	14	50	93	100	100
Plain agar made with distilled water:						
Sewage	9	40	41	59	59	59
Filtered sewage	0	0	58	81	99	100
Merrimack River.....	16	37	42	58	58	58
Filtered water.....	7	14	14	14	14	21

water, Lawrence tap water, *i. e.*, Merrimack River water after filtration through a slow sand filter; distilled water, *i. e.*, the first portion distilled from a large still, this containing considerable free ammonia, but no solids. The chemical analyses of the waters used are shown in Table III.

Samples of the waters used in the preparation were saved, and plated out on the various media. With sewage the maximum count was obtained on the agar made with sewage. The maximum growth with the polluted river water and with the filtered water was obtained on the agar made with filtered water, while the filtered sewage, the purest water of all from a bacteriological standpoint, showed the best development on the agar made with distilled water. Both filtered water and filtered sewage failed to show any growth on media made with sewage. Other samples of water of the same classes followed the same general laws as to preference as did the waters with which the media were made. The relative numbers of bacteria with the same waters used in the preparation of the various media are shown in Table IV.

EFFECT OF THE SALTS PRESENT IN COMMERCIAL AGAR ON THE DEVELOPMENT OF BACTERIA.

It has been pointed out that one of the sources of irregularity in the use of agar media has probably been the variation in the natural salt content of the crude agar. In order to test this phase of the problem, a lot of commercial agar was divided in two portions, one portion of which was used to make plain agar, the other portion being allowed to soak over night in a 5 per cent. solution of glacial acetic acid* in distilled water, and then washed three to four hours in running water, after which plain agar was made as with the first portion. The theoretical effect of the acetic acid would be to decompose the compounds of organic acids with the alkaline bases, forming soluble compounds which would readily be washed out. No analysis was made to determine what proportion of the salts was removed by this process.

Different classes of water were plated on the two media, and daily counts made as usual until a maximum count was obtained.

*The treatment with acetic acid was the suggestion of Dr. George T. Moore.

The development of bacteria was slightly better on the agar from which the salts had been removed than on the commercial agar, although with two classes of waters the commercial agar gave higher numbers than the purified agar. The results of comparisons of these two media are shown as follows in Table V:

TABLE V.

Relative Numbers of Bacteria on Plain Agar Made with Commercial Shred Agar and with Washed Commercial Shred Agar.

CLASS OF WATER	COMMERCIAL AGAR				WASHED AGAR			
	2 Days	4 Days	6 Days	8 Days	2 Days	4 Days	6 Days	8 Days
Sewage	13	15	29	31	27	65	100	100
Filtered sewage	36	91	91	91	22	80	99	100
Merrimack River	42	71	97	100	17	55	94	96
Filtered water	40	40	85	100	37	38	67	73
All waters	35	57	80	85	27	63	95	100

EFFECT OF GLYCERIN.

Considerable contention has arisen in the past as to whether it was a benefit to use a glycerinated agar for bacterial counts. We know that there are certain forms of bacteria which will grow on a glycerinated medium, but will not grow on standard agar. These forms are, however, probably never met with in water analysis, and their behavior should not be considered in this connection. Until within about two years an agar containing 3 per cent. of glycerin was the regular medium used at Lawrence, the glycerin being omitted from the agar at the time the methods were changed, to agree with the recommendation of the Bacteriological Committee. The use of glycerin agar in preference to agar without glycerin was based on experiments made in the early history of the Experiment Station, which data have been accidentally destroyed. Studies have been made with agar containing various percentages of glycerin, which show that somewhat better development is obtained on media without the glycerin. With the majority of waters, however, variations in the amount of glycerin have caused only a comparatively slight variation in the relative numbers. With filtered sewage the best results were obtained on

a medium containing 6 per cent. of glycerin, the medium containing 1 per cent. of glycerin being a close second, and the medium containing no glycerin coming third. With sewage there was a maximum deviation of only 1 per cent. between media containing no glycerin and any of the media containing glycerin. With water polluted by sewage this deviation was considerably greater, while with filtered water approximately the same development occurred on the medium containing 2 per cent. of glycerin and on the medium containing no glycerin. The results of the determinations on these media are shown as follows in Table VI:

TABLE VI.

Relative Numbers of Bacteria on Beef-Pepton Agar Containing Varying Amounts of Glycerin.

Class of Water	Percentage of Glycerin						
	0	1	2	3	4	5	6
Sewage.....	100	96	98	93	95	96	93
Filtered sewage....	83	96	68	72	59	68	100
Merrimack River..	100	94	83	68	62	75	64
Filtered water.....	100	72	100	37	77	69	62
All waters.....	100	94	90	71	77	81	86

STUDIES OF NÄHRSTOFF AGAR.

In the previous paper it was shown that higher counts of bacteria were obtained with Nährstoff agar than with any other medium, and it was stated that this was probably due to the fact that certain species of bacteria which do not grow on the usual media do flourish on this medium, and that many debilitated individual bacteria which have not the vitality to produce colonies on the usual media are able to develop slowly on this medium. In order to test the truth of this last statement, experiments have been made with pure cultures of different species of bacteria. Three lines of investigation have been carried out with each. The cultures were put through three generations in broth, then transferred to agar streaks, and, after a two-day incubation, water suspensions were made of the surface growth. After being thoroughly shaken, these were plated out on Lawrence agar, standard gelatin,

and Nährstoff agar. The water suspensions were then placed on ice, and allowed to stand two days, after which they were thoroughly shaken and again plated on the three media. Water suspensions were also made from agar slants on which the growth was 30 days old, and plated as before. These three methods gave a check upon one another the first giving us as nearly as possible all active bacteria; the second, the condition where the bacteria had been subjected to an unfavorable state; and the third, a case where the majority of the individual bacteria were debilitated by the length of time they had stood on media. The cultures were selected to cover as many types of water bacteria as possible, in all eighteen cultures being studied.

The results show that our former belief that certain debilitated individuals would develop on Nährstoff agar when they would not develop on standard gelatin or agar, was not well grounded, in nearly every case the counts on the Nährstoff agar being less than on the other two media, and in the majority of cases the counts on

TABLE VII.

Relative Development of Pure Cultures of Bacteria on Standard Gelatin, Lawrence Agar, and Nährstoff Agar.

Group*	Name*	Water Suspension from Fresh Culture			Water Suspension after Two Days on Ice			Water Suspension from Thirty- Day-Old Culture		
		Gelatin	Agar	Nährstoff Agar	Gelatin	Agar	Nährstoff Agar	Gelatin	Agar	Nährstoff Agar
1129	Sewage streptococcus No. 131	100	95	1	100	100	0	100	123	0
1129	Sewage streptococcus No. 191	100	72	0	100	79	0	100	90	0
1229	Micrococcus No. 111	100	84	90	100	64	78
1354	Sarcina Lutea No. 26	100	145	0	100	43	2	100	53	14
2139	Bacterium No. 110	100	83	91	100	83	100	100	100	90
2139	Bacterium No. 116	100	57	21	100	44	33	100	72	19
2139	Bacterium No. 117	100	82	76	100	100	100	100	94	100
2269	Pseudomonas No. 126	100	80	75	100	100	97	100	166	230
2429	B. typhosus No. 143	100	99	42
2429	B. typhosus No. 145	100	62	20
2429	Bacillus No. 89	100	59	56	100	80	60	100	90	21
2429	B. intestinalis No. 128	100	80	96	100	86	77	100	83	50
2439	B. coli No. 192	100	38	37	100	35	22	100	46	64
2439	Bacillus No. 51	100	83	84	100	92	75	100	80	32
2439	Bacillus No. 56	100	74	75	100	37	87	100	53	0
2439	Bacillus No. 58	100	75	67	100	63	18	100	55	25
2439	Bacillus No. 107	100	92	74	100	98	59	100	50	89
2449	Bacillus D (Bact. comm.)	100	64	61	100	58	50
2469	Bacillus B (Bact. comm.)	100	65	29	100	28	5	100	176	62

* For descriptions of Lawrence Species and methods of grouping see *Rept. Mass. Board of Health*, 1901, p. 415.

gelatin being the highest. With the old cultures a greater relative number of bacteria developed on the Nährstoff agar than with the fresh cultures. It may be, however, that our range of cultures did not include the specific types which would cause the increased counts on the Nährstoff agar with ordinary waters.

Some attempts have also been made to prove that species naturally present in water, which would not grow on the usual gelatin or agar, were responsible for the increased counts on the Nährstoff agar. To show this, a large number of colonies from the Nährstoff agar plates have been transferred to Nährstoff agar streaks, and the growth from these have been tested to see whether the species would grow on ordinary gelatin and agar. So far, however, these results have proved very unsatisfactory, and have not given us the information we desired.

The results of the counts of the three types of cultures with the different species are shown in Table VII.

THE EFFECT OF DECREASING THE AMOUNT OF NÄHRSTOFF IN NÄHRSTOFF AGAR.

The fact that a reduction in the amount of nutrients in the usual media allowed of an increase in the bacterial counts led us to believe the same might be true with Nährstoff, and that a better growth would be obtained on media containing less than

TABLE VIII.

Relative Numbers of Bacteria on Nährstoff Agar of Different Strengths.

Class of Water	Percent- age of Nährstoff	2 Days	4 Days	6 Days	8 Days	10 Days
Sewage	1.0	10	19	25	25	25
	0.5	45	59	97	100	100
Filtered sewage ..	1.0	20	35	35	43	43
	0.5	35	89	96	100	100
Merrimack River .	1.0	14	41	55	55	55
	0.5	14	47	83	100	100
Filtered water....	1.0	2	29	45	54	59
	0.5	12	88	94	100	100
Lake water.....	1.0	0	4	7	13	13
	0.5	5	81	100	100	100
All waters....	1.0	9	25	33	38	39
	0.5	22	73	94	100	100

1 per cent. of Nährstoff. Two lots of Nährstoff agar were made, one containing 1 per cent. as usual, and the other one-half this amount (0.5 per cent.); and comparative platings were made on the two media with the various classes of water. In every case an increased number of bacteria appeared on the medium which contained the smaller amount of Nährstoff. The results of these experiments are shown in Table VIII.

EFFECT OF COOKING NÄHRSTOFF AGAR IN OTHER THAN NEUTRAL SOLUTION

It is well known that better results have been obtained in the preparation of ordinary culture media if the cooking were done in a solution which was slightly alkaline, and the usual methods of preparing standard gelatin and agar have allowed for this fact. It is believed that the reason for this was the breaking down of many of the albumens, either into albumens of less complex structure or into simpler compounds. Nährstoff being a compound of albumens and albumoses, it was reasonable to believe that the method of preparing in neutral solution might not be the procedure best adapted to obtain the most favorable medium for bacterial development. Three lots of Nährstoff agar were made of the same percentage composition. All of these were allowed to cook slowly for one hour on the water bath, one of them being unchanged by the addition of acid or alkali, to one of them sufficient normal potassium hydrate being added to make the solution about 1 per cent. alkaline, and to the third sufficient normal hydrochloric acid being added to make the reaction about plus 1 per cent. After cooking, acid or alkali was added in sufficient amount to bring them all back to the neutral point, and they were then filtered, tubed, and sterilized as usual. Titrations made after sterilization showed that the three media were of the same reaction. Comparative platings were then made on the three media with waters of different classes. As had been expected, the media made in alkaline solution gave higher counts with four out of five waters. With sewage the media made in acid solution gave the highest counts. The results obtained with the different classes of water on these media are shown as follows in Table IX:

TABLE IX.

Relative Numbers of Bacteria on Nährstoff Agar of the Same Strength Made by Different Procedures.

Class of Water	Procedure	2 Days	4 Days	6 Days	8 Days	10 Days
Sewage	Neutral	25	46	62	62	62
	Alkaline	42	59	71	85	85
	Acid	47	82	100	100	100
Filtered sewage.....	Neutral	30	53	54	54	54
	Alkaline	44	70	89	100	100
	Acid	33	49	67	73	73
Merrimack river.....	Neutral	14	37	50	54	54
	Alkaline	10	68	92	100	100
	Acid	10	69	81	92	95
Filtered water.....	Neutral	2	31	49	59	66
	Alkaline	12	64	89	100	100
	Acid	17	42	55	58	58
Lake water.....	Neutral	0	4	6	11	11
	Alkaline	5	41	71	98	100
	Acid	4	59	82	82	82
All waters.....	Neutral	15	35	46	50	51
	Alkaline	23	62	85	100	100
	Acid	23	62	80	84	84

THE EFFECT OF VARIATION IN MEDIA ON THE DETERMINATION OF THE EFFICIENCY OF WATER FILTERS IN THE REMOVAL OF BACTERIA.

The principal use of counts of bacteria at the Experiment Station has been in the determination of the numbers of germs in the applied waters and effluents from the various water filters, and it has always been assumed that, although the different lots of media vary among themselves, the ratio between the numbers in the applied waters and in the effluents would be approximately a constant on media of the same kind; in other words, that we had comparative results. The use of agar instead of gelatin for work of this class has always been thought preferable, since it has been proved that agar was less subject to variation than gelatin. It has always been the practice at Lawrence to compare directly each lot of agar and of gelatin with the preceding lot and the agar and the gelatin, gelatin being the standard medium in so many other laboratories that our idea was to approximate the counts on the agar to those which would be obtained on the standard gelatin. In each comparison we have plated all the classes of water at the Experiment Station, and from comparisons made during 1902 we

have traced out the variation in the efficiency of some of the water filters, as determined by the counts on agar and by those on gelatin. In thirty-nine comparisons during the year an applied water for, and an effluent from, a filter were plated on the various lots of media from which we could figure the efficiency of the filter for the day. In a considerable number of comparisons the efficiency, as figured by two or more agars, proved to be the same, but in no case was it found that two lots of gelatin gave the same efficiency figure, the minimum deviation between any two lots of gelatin being 0.10. The results of the estimation of efficiency by different lots of gelatin and agar were as follows:

Average maximum deviation between different gelatins	-	-	-	-	0.92
Average maximum deviation between different agars	-	-	-	-	0.53
Maximum deviation between different gelatins	-	-	-	-	4.50
Maximum deviation between different agars	-	-	-	-	5.00
Minimum deviation between different gelatins	-	-	-	-	0.10
Minimum deviation between different agars	-	-	-	-	0.00
Average deviation of agar from gelatin	-	-	-	-	+0.09
Number of times gelatin greater than agar	-	-	-	-	16
Number of times agar greater than gelatin	-	-	-	-	18
Number of times agar and gelatin were the same	-	-	-	-	50

EFFECT OF COMPOSITION OF MEDIA AND PERIOD OF INCUBATION ON THE CALCULATED EFFICIENCY OF WATER FILTERS.

In a number of comparisons of the various media which we have studied we have plated an applied water and an effluent on the different media on the same day, and we have thus been able to calculate and compare the efficiency as determined by the various media and after different periods of incubation. In Table X four such comparisons are shown. The difference between the efficiency, as determined on various media by counts on different days, has been relatively small in many instances; still the maximum difference has been sufficient, if the variation occurred in routine work, to cause a false opinion as to the quality of the work the filter was doing.

The whole question of efficiency is based on the assumption that the ratio between the determined number of bacteria and the total number of any medium is a constant, for both applied water and effluent should be the same. If a greater percentage of the

total number of bacteria in the applied water develop on a given medium than was the case with the effluent, we should obtain an efficiency value which was too high; and, on the other hand, if the reverse be true, we should obtain an efficiency value which was too low. In Table XI are shown the relative numbers of bacteria on a raw water and the effluents from two water filters (A and B), on agar of various reactions; and also the various efficiency values for these two filters as calculated from the actual counts. The effect

Variation in the Percentage Removal of Bacteria by Water Filters when
Estimated by Counts on Different Media and after Different Periods
of Incubation.

MEDIA	SERIES I				SERIES II			
	Two Days	Four Days	Six Days	Eight Days	Two Days	Four Days	Six Days	Eight Days
Plain agar	99.9	99.9	99.8	99.7	98.7	98.7	98.9	98.8
Pepton agar
Beef agar	99.8	99.9	99.9	99.9	98.8	98.7	98.9	98.9
Lawrence agar	99.8	99.8	99.8	99.8	98.8	99.1	99.2	99.2
Plain gelatin
Pepton gelatin
Beef gelatin
Standard gelatin
Nährstoff agar	99.7	99.0	99.0	99.0	93.8	96.8	96.8	96.8
Nährstoff pepton agar	99.8	99.5	99.5	99.5	98.6	98.0	98.1	98.1
Nährstoff beef agar	99.9	99.5	99.3	99.4	98.4	98.7	98.7	98.7
Nährstoff glycerin agar	100.0	98.8	99.8	99.0	99.0	97.7	97.9	97.9

[illegible]

of the change in percentage of bacteria developing on the various media on the calculated efficiency is well shown.

TABLE XI.

Relation between Numbers of Bacteria and Efficiencies of Water Filters with Agar of Different Reactions.

REACTION	RELATIVE NUMBERS OF BACTERIA			PERCENTAGE OF BACTERIA REMOVED	
	Raw Water	Filtered Water		A	B
		A	B		
-1.5.....	20	74	29	99.0	99.5
-1.0.....	90	81	44	99.7	99.8
-0.5.....	32	100	61	99.1	99.3
0.....	84	68	63	99.8	99.7
+0.6.....	65	88	63	99.6	99.6
+1.2.....	37	48	86	99.6	99.1
+1.6.....	100	93	100	99.7	99.6
+2.1.....	50	74	54	99.6	99.6
+2.6.....	2	6	7	99.0	98.5

TABLE XII.

Variation in Efficiency of Lawrence City Filter when Estimated by Counts of Bacteria on Agar of Different Reactions.

Sample Number	Reaction			
	-1.0	0.0	+1.0	+2.0
1.....	98.9	98.1	98.4	98.8
2.....	98.6	99.0	99.3	99.0
3.....	98.7	99.0	99.1	99.6
4.....	98.2	98.7	98.7	99.1
5.....	99.2	99.1	99.0	99.2
6.....	96.5	96.7	97.7	98.5
7.....	98.2	99.0	98.9	99.4
8.....	99.1	98.8	98.7	98.5
9.....	97.8	97.5	98.3	98.8
10.....	99.5	98.8	98.5	98.3
11.....	99.9	99.9	99.9	100.0
12.....	88.8	88.8	94.5	93.6
13.....	99.1	98.3	98.7	99.1
14.....	98.8	98.3	98.8	99.2
15.....	99.3	97.1	98.4	98.9
16.....	97.4	96.0	97.9	97.9
17.....	99.2	99.3	99.5	99.4
18.....	99.4	99.6	99.7	99.4
19.....	96.6	99.3	97.8	95.8
Average.....	98.1	97.9	98.5	98.5
Percentage of times maximum.....	21	5	32	42

In order to test the constancy of the determination of the efficiency on agar of different reactions, samples from the effluent of the Lawrence City filter and the applied water for that filter were plated on such media nineteen times during one month. The highest average efficiency determined during this period was 98.5, that being the same on two different reactions. The maximum variation, however, on the agar with the reaction of 1 per cent. was much less than was the variation on the other three media. The optimum reaction used for ordinary work at Lawrence is about 1.4 per cent. The results of this series of comparisons are shown in Table XII.

NOMENCLATURE OF MEDIA.

Much confusion has been caused in the past through the use of the same terms for the usual media and for special media. The recommendation of the Bacteriological Committee, that media made according to its formulæ should be specifically labeled B. C., has not been followed in a number of instances, various names being applied to media of the same composition. One example from our own experience will illustrate this: About a year ago we had occasion to repeat certain experiments of a well-known bacteriologist, and, after an investigation extending over some weeks, we found that we were unable to obtain the phenomena which he described as being both constant and characteristic. Correspondence with the original observer revealed the cause of our trouble to be in the different composition of the media used. He had stated that the base for his medium was "plain agar," meaning "standard agar, B. C.," in distinction to glycerin agar, which he used in another process; while we had used a solution of agar in water without the addition of the usual nutrient materials, this being the "plain agar" generally understood.

In a former contribution we used certain names as descriptive of our media, these names indicating in nearly every case the ingredients used in the preparation of the medium; and, furthermore, we made a distinct statement of the exact composition and method of preparation of the various media used. In the present transitional stage of bacteriological methods some such exact

expression seems necessary, that confusion may be avoided, and every attempt should be made to have such nomenclature as concise and descriptive as possible, and to have a uniform set of names for all standard and special media.

The following elementary rules are suggested as a basis for such nomenclature :

1. That liquid media in which the solvent is water be called "solutions," as, for example, "pepton solution" or "Dunham's solution," *i. e.*, pepton dissolved in water.

2. That liquid media in which the solvent is beef-infusion be called "broths," as, for example, the usual "standard broth" or "pepton broth," *i. e.*, pepton dissolved in beef-infusion.

3. That a distinction be made between media made with beef-infusion and that made with commercial beef extracts, the former being the standard ingredient, its use to be understood unless the use of the commercial extracts is distinctly stated.

4. That all media made after the formulæ of the Bacteriological Committee either have the prefix "standard," as "standard gélatin," or else be followed by the letters "B. C.," as "gelatin B. C."

5. That all media in regular use in any laboratory which approximate the "standard" or "B. C." media in composition or method of preparation, and which are substituted for the latter, be preceded by the name of the laboratory or of the individual, as "Lawrence agar," "Wurtz agar," etc.; or else be known by a name combining the various ingredients, as "pepton agar," *i. e.*, agar and pepton in water, or "plain agar," *i. e.*, agar in water.

6. That the names of special media express, as far as possible, the composition, *i. e.*, the ingredients used in their preparation.

7. That when media of standard composition are used, which vary only in reaction, this reaction should be distinctly stated, preferably in parentheses in the name, as "standard agar (+2.0)" or "agar (+2.0) B. C."

8. That when other brands of commercial pepton are used than Witte's, the same be distinctly stated, preferably by the insertion of the name of the brand in parentheses in the name, as "pepton (Merck) agar."

9. That the use of the word "nutrient" as signifying "containing pepton and beef-infusion" has become so general that it may possibly be retained without any resulting confusion, although, strictly speaking, its use is improper, since a distilled water has sufficient nutrient properties to allow of an abundant bacterial development, to say nothing of a solution of gelatin in water without the addition of beef-infusion and pepton.

CONCLUSIONS.

In standard gelatin or agar the beef broth is the most fertile source of variation, the amount of nutrient matter in beef-infusion made by the usual method varying through wide limits.

Of the two best-known commercial peptons, Merck's and Witte's, higher bacterial counts are obtained on media made with the latter.

Studies of plain agar made with various kinds of natural water show that in the majority of cases the bacteria naturally present in the given water will develop in greatest numbers in a medium made with that same water.

The salts naturally present in commercial agar have a detrimental effect, greater numbers of bacteria developing on media made with agar from which the natural salts have been washed out.

Greater numbers of bacteria will develop on media made without glycerin than on the same media containing glycerin.

With pure cultures of different kinds of bacteria on standard gelatin, agar, or Nährstoff agar, the highest counts were obtained, with the species studied, on gelatin, and the lowest on Nährstoff agar.

A reduction of the amount of Nährstoff in Nährstoff agar from 1.0 per cent. to 0.5 per cent. results in a considerable increase in the bacterial counts with various waters.

When cooked in alkaline solution, the albumoses of Nährstoff undergo certain changes in composition which render them better food material for bacteria than is the commercial product.

Studies of the efficiency of water filters in removing bacteria, when estimated by counts on different media, show that there is a considerable variation between the various media, the ratio

between the numbers of bacteria in the raw water and in the effluent being affected by the period of incubation, the reaction, and the composition of the media. With standard gelatin and Lawrence agar the ratio was less variable between different lots of agar than between different lots of gelatin.

The writers believe that it is time a uniform system of nomenclature were adopted, and that the names applied to the various media should convey definite information as to the composition, method of preparation, etc., of those media.

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THE ANTILYTIC ACTION OF SALT SOLUTIONS AND OTHER SUBSTANCES.*

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INTRODUCTION.

SINCE the earliest bacteriolytic experiments with blood serum, solution of sodium chloride has been used as a diluent; and in the more recent experimental work with lysins of various kinds, as well as with agglutinins and precipitins, the same solution has been used universally for making the necessary dilutions of the mixtures employed. No reason whatsoever has arisen to question in any way the general usefulness of common salt solution for these purposes. Sodium chloride solution of approximately the same concentration as human blood serum is in itself, as a rule, not bacteriolytic or cytolytic; it appears to maintain intact and active the various lysins in normal and immune sera, in venoms, and in other substances; and it does not appear in any noteworthy degree to hinder their union with the different cells and bacteria used.¹ In order to test the action of other chemical substances, especially the various salts (ions) that occur in the normal plasma,

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¹ MADSEN AND ARRHENIUS (*Festskrift ved Indvielsen af Statens Serum-Institut*, Copenhagen, 1902) found that NaCl solution decreases the hemolytic action of bases, and to a less extent of tetanolysin.

upon the bodies concerned in lysis, one can substitute in the ordinary experiments for solution of sodium chloride varying quantities of solutions of other salts of nearly the same concentration ($\frac{m}{8}$) as the so-called physiological salt solution.

In this way important facts might come to light. Substances might be found that diminish or modify in some way the activities of lysins, and the precise mode of action of such substances could be studied. Furthermore, the physico-chemical behavior of the different elements concerned in lysis might perhaps be made clear to some extent, and some light might be thrown upon the intricate mechanisms that lead to diminution or suspension of the lytic properties of serum under various conditions. With these and other considerations in mind, the effect of a number of solutions was tested upon the hemolytic action of human serum on rabbit corpuscles.¹ It was shown that the chlorides of calcium, barium, strontium, magnesium, and the sulphates of magnesium and potassium suspend hemolysis by their action on the complement when 0.2–0.4 c.c. of $\frac{m}{8}$ solution are allowed to act upon 0.2 c.c. of serum. The solutions used being ionizable, it was suggested that this antihemolytic action might be due to the bivalent ions present. It was also pointed out that the same salt solutions suspend the bacteriolytic effect of human serum upon typhoid bacilli. Manwaring² then studied the effect of certain salts upon the complement in the serum of a goat immunized with dog blood. He shows that CaCl_2 , BaCl_2 , MgCl_2 , Na_2SO_4 , $\text{Na}_2\text{C}_2\text{O}_4$, and $\text{Na}_2\text{C}_2\text{H}_4\text{O}_6$ inhibit the lytic action of this serum on dog corpuscles; that the amount of salt necessary to bring about this inhibition varies with the amount of complement in the serum; and that precipitation of the salts from non-hemolytic serum salt mixtures restores the original hemolytic power, which may be destroyed again by heating the fluid to 56°C . for forty minutes. For these reasons Manwaring is inclined to believe that the antilytic action of the salts mentioned is due to the formation of simple salt- or ion-complement compounds that are hemolytically inactive.

¹ HEKTOEN, *Trans. Chicago Path. Soc.*, 1903, 5, p. 303.

² *JOUR. OF INFECT. DIS.*, 1904, 1, p. 112.

In this article we intend to present further and more diversified observations upon the action of a somewhat larger list of salt solutions on the lytic substances in a variety of sera, with the special view to establish to what extent the antilytic action of certain salts holds true of sera in general, and also, if possible, to throw further light upon its mechanism.

BRIEF REVIEW OF NON-SPECIFIC ANTILYSINS.

Various substances other than specific antibodies have been found to diminish or suspend the lytic action of serum and other lysins. Nissen long ago observed that the addition of MgSO_4 to plasma in order to hinder coagulation renders the plasma non-bactericidal. Behring¹ found that oxalic acid rendered rat serum harmless for anthrax bacilli because, so he thought, the alkalinity of the serum was reduced. Von Lingelsheim² observed that the "alexin" is variously affected by different salts. While KNO_3 , when added to rabbit serum in the proportion of 2.5 per cent., had little effect on its destruction of anthrax bacilli, CaCl_2 and $(\text{NH}_4)_2\text{SO}_4$ had a marked inhibitive action. Ferric sulphate, alum, and tannin also reduced the bactericidal power of the serum. He showed, too, that any increase in the salt content of serum reduces its bactericidal power, no matter whether the increase is the result of evaporation or due to the direct addition of salts. Not only an increase in NaCl has this effect, but KNO_3 , K_2HPO_4 , Na_2SO_4 , $(\text{NH}_4)_2\text{SO}_4$, and CaCl_2 , when added to rat serum in the proportion of 2 per cent., suspended its bactericidal action on cholera vibrios. Noting the similarity in this effect of salts to their effects upon certain ferments, he suggested that possibly this antibactericidal action is due to the abstraction of water, a certain amount of which is essential for the function of ferments. Markl³ showed that concentrated salt solutions prevent hemolysis. He regarded this as due to changes in the cell membrane; but Ehrlich and Sachs,⁴ while confirming the observation, interpret the antilytic action as due to failure of complement to unite with the receptor which is bound by the corpuscles. They point out that Knorr has shown that 10 per cent. NaCl solution prevents the union of tetanus toxin with antitoxin. Here may be mentioned that Bail and Pettersson⁵ find that rabbit serum treated with suspensions, or extracts of organs (liver, kidney, muscle), is deprived of its bactericidal action upon anthrax bacilli.

V. Dungern⁶ removed the hemolytic complement from serum by shaking it with emulsions of bacteria, with powdered yeast, and with the cells of various organs. Ehrlich and Sachs⁷ also found yeast to remove complement

¹ *Gesammelte Abhandl.*, Leipzig, 1893.

² *Ztschr. f. Hyg. u. Infektionskrankh.*, 1901, 37, p. 131.

³ *Ibid.*, 1902, 39, p. 86.

⁴ *Berl. klin. Wchnschr.*, 1902, 39, p. 492.

⁵ *Centralbl. f. Bakteriol.*, 1903, I. Abt., 34, p. 445 ff.

⁶ *Münch. med. Wchnschr.*, 1900, 47, p. 677.

⁷ *Berl. klin. Wchnschr.*, 1902, 39, pp. 297, 355.

from serum. Wilde¹ found that mixing sera with heated bacterial emulsions (anthrax, cholera, typhoid) and with insoluble albuminous substances such as aleuronat removed the bactericidal and hemolytic qualities of the serum. V. Lingelsheim² shows that the filtered decoction of Irish moss (carrageen or carrigeen, *Chondrus crispus*, and *Gigartina mamilliosa*) when added to serum carries down both complement and intermediary body, especially when the alkalescence of the serum is reduced. Hoke³ shows that organ cells and leucocytes bind the complement in hemolytic serum. Kraus and Lipschütz⁴ find that the saline extracts and filtrates of normal organs have the power to bind various hemolysins (megatheriolysin, the lysin of vibrio Naskin, staphylolysin).

The antitetanolytic action of horse serum and egg albumin was explained by Arrhenius and Madsen⁵ as due to a union between the tetanolysin and the proteids in the serum. Müller,⁶ on the other hand, holds that the anti-hemolytic substances cannot be proteid in character because soluble in alcohol. He favors the idea that the antihemolytic action is due to cholesterol, which has been shown by Ransom⁷ to prevent hemolysis by saponin and to give normal serum its antisaponin action, and by Noguchi⁸ to be antitetanolytic—a form of antitoxic action which Bashford has designated as pseudoantitoxic. Madsen and Arrhenius found that neutral salts decrease the hemolytic action of bases and to a less extent of tetanolysin. Normal serum and egg albumin also diminish the lytic action of bases. They explain the antilytic action of salts upon bases by assuming that the combination of red corpuscle and base behaves as a weakly dissociated salt, of which only the dissociated molecules are active, and the dissociation of which is decreased by the presence of neutral salt. Bullock⁹ found that solutions of $MgSO_4$, $K_2C_2O_4$, and a number of other salts, isotonic with blood corpuscles, greatly reduced the lytic power of immune rabbit serum. Special experiments with $MgSO_4$ seemed to show that lysis was inhibited because the union of complement with amboceptor was prevented.

Wendelstadt¹⁰ shows that glycogen suspends or diminishes hemolysis by its influence on the complement, but only when the amount of amboceptor present is small. For this reason the antihemolytic action of glycogen is demonstrable only in normal serum, and not in immune serum which contains amboceptor in excess. Furthermore, the complement is bound by glycogen only when the latter is added to serum before the proper corpuscles. He states that inulin and peptone diminish hemolysis, the former in normal and immune serum. It is of great interest to note that the antihemolytic

¹ *Ibid.*, 1901, 38, p. 878; *Arch. f. Hyg.*, 1902, 39, p. 404.

² *Ztschr. f. Hyg. u. Infektionskrankh.*, 1903, 42, p. 308.

³ *Centralbl. f. Bakteriöl.*, 1903, I. Abt., 34, p. 692.

⁴ *Wien. klin. Wchnschr.*, 1903, 16, p. 989.

⁵ *Loc. cit.*

⁶ *Centralbl. f. Bakteriöl.*, I. Abt., 1903, 34, p. 567.

⁷ *Deutsche med. Wchnschr.*, 1901, 27, p. 194.

⁸ *Centralbl. f. Bakteriöl.*, I. Abt., 1902, 32, p. 377.

⁹ *Trans. Lond. Path. Soc.*, 1903, 54, p. 260.

¹⁰ *Centralbl. f. Bakteriöl.*, 1903, I. Abt., 34, p. 831.

action of glycogen may be exercised *in vivo*, the normal hemolytic power returning gradually, reaching the normal in seven days after injecting 3 g. of glycogen into the ear vein of rabbits in three equal doses during twenty-four hours.

We do not intend to refer in detail to the presence of antilysins in normal sera, but mention must be made of the power of uremic serum to inhibit hemolysis by normal human serum noted by various observers.¹ It would be interesting to learn whether the scope of the inhibition extends farther than to human serum, and, if so, the exact mechanism.

THE ACTION OF SALT SOLUTIONS UPON BACTERIOLYSIS BY NORMAL SERA.

In order to test the effect of salt solutions upon the bacteriolysins in blood serum, the following experiments were made: $\frac{m}{8}$ solutions of the best chemicals obtainable were made in pure, redistilled water, kept in carefully cleansed glass bottles, and used in sterile condition for diluting the serum, as in the usual experiments for the purpose of demonstrating the destructive effect of human serum upon typhoid bacilli. A fixed quantity of twenty-four-hour broth cultures of recently isolated typhoid bacilli, dysentery bacilli, or anthrax bacilli was introduced into a constant quantity of the serum mixture, and the number of bacilli estimated from time to time by counting the colonies that developed on agar plates made with a fixed quantity of the mixtures. The mixtures of serum and solutions of salts were allowed to stand for about one hour before adding the bacilli. This method gives one a fairly satisfactory idea of the fate of the bacilli, but, of course, the chances for error are too great to warrant any attempt to determine accurately minor comparative differences in bactericidal action. The results are set forth in Tables I and II.

Table I shows that even small quantities of many salts are capable of completely inhibiting the lytic action of human serum upon typhoid bacilli and dysentery bacilli. The best effect is obtained with salts of the alkaline earths, but we find here that $MgSO_4$ forms an exception to the rule. $MgSO_4$ is not strongly germicidal, and hence the death of the bacilli introduced into

¹CAMUS AND PAGNIEZ, *Soc. de Biol.*, 1901, 53, p. 730; NEISSER AND DOEHRING, *Berl. klin. Wchnschr.*, 1901, 38, p. 593; LACQUER, *Deutsche med. Wchnschr.*, 1901, 27, p. 744; HAHN AND TROMSDORF, *Münch. med. Wchnschr.*, 1902, 49, p. 1454; HEDINGER, *Deutsche Archiv f. klin. Med.*, 1902, 74, p. 24; WENSTRAND, *Trans. Chicago Path. Soc.*, 1903, 5, p. 288.

the mixture of serum and a $\frac{m}{8}$ solution of this salt cannot be ascribed to that property of the salt. It was found also that $\frac{m}{8}$ solutions of some salts, as BaCl_2 , KCNO , NaHCO_3 , and $\text{Na}_2\text{HC}_4\text{H}_4\text{O}_6$, are germicidal when used alone, while a mixture

TABLE I.

THE EFFECT OF SALTS ON LYSIS OF TYPHOID AND DYSENTERY BACILLI BY NORMAL HUMAN SERUM.

B. TYPHOSUS				B. DYSENTERIAE			
$\frac{m}{8}$ Solutions		At Once	4-5 Hours	$\frac{m}{8}$ Solutions		At Once	4-5 Hours
NaCl	0.8	5,000	0	NaCl	0.8	20,000	0
CaCl ₂	0.4	3,600	1,800	CaCl ₂	0.4	20,000	∞
"	0.2	3,800	1	"	0.2	20,000	5,000
BaCl ₂	0.2	7,000	6,000	BaCl ₂	0.2	12,000	8,000
SrCl ₂	0.4	6,000	7,000	SrCl ₂	0.4	20,000	∞
"	0.2	5,500	5,000	"	0.2	20,000	∞
MgCl ₂	0.4	4,500	3,500	MgCl ₂	0.4	12,000	10,000
"	0.2	4,500	0	"	0.2	12,000	1,200
MgSO ₄	0.4	2,000	0	MgSO ₄	0.4	12,000	90
"	0.2	2,000	0	"	0.2	12,000	200
(NH ₄) ₂ SO	0.4	4,500	200	(NH ₄) ₂ SO ₄	0.4	12,000	8,000
K ₂ SO ₄	0.4	3,800	0	K ₂ SO ₄	0.4	10,000	10,000
NaHCO ₃	0.2	4,300	2,500	NaHCO ₃	0.2	12,000	12,000
"	0.1	3,500	3,500	"	0.1	12,000	220
Na ₃ C ₆ H ₅ O ₇	0.4	3,500	1,800	Na ₃ C ₆ H ₅ O ₇	0.4	12,000	∞
"	0.2	4,000	1	"	0.2	12,000	10,000
Na ₂ C ₂ O ₄	0.4	3,800	3,500	Na ₂ C ₂ O ₄	0.4	12,000	15,000
"	0.2	3,500	11	"	0.2	12,000	15,000
(NH ₄) ₂ C ₂ O ₄	0.4	5,000	5,500	(NH ₄) ₂ C ₂ O ₄	0.4	20,000	∞
"	0.2	5,000	12	"	0.2	20,000	∞
K ₄ Fe(CN) ₆	0.4	3,500	4,000	K ₄ Fe(CN) ₆	0.4	20,000	∞
"	0.2	3,500	4,000	"	0.2	20,000	∞
K ₆ Fe ₂ (CN) ₁₂	0.4	5,500	55	K ₆ Fe ₂ (CN) ₁₂	0.1	20,000	12,000
KCNO	0.4	3,500	2,500	KCNO	0.4	12,000	10,000
"	0.2	3,500	2,500	"	0.2	12,000	12,000
NaHC ₄ H ₄ O ₆	0.4	3,500	1,800	NaHC ₄ H ₄ O ₆	0.4	12,000	15,000
"	0.2	4,000	4,000	"	0.2	12,000	15,000

NOTE.—Each tube contained 0.2 c.c. of human serum + 0.1-0.4 c.c. $\frac{m}{8}$ solution of salt + 0.85 per cent. sodium chloride solution enough to make 1 c.c. The experiments represented by the figures in the right-hand columns of both Tables I and II were made as follows: Each tube was inoculated with a loopful of a twenty-four-hour broth culture, and at the same time several plates were made each with one loopful. The average number of colonies in these plates was taken as the number of organisms inoculated into each tube (see "At Once" column). In from four to five hours the entire contents of each tube were plated and the colonies counted in twenty-four hours. In the left-hand columns of Tables I and II each tube was inoculated with a loopful of culture, and two loopfuls of this mixture were plated at once and in four to five hours. Twenty-four plates were also made in many cases, but these showed in reality nothing in addition to what is shown by the five-hour plates; in most instances the bacilli had multiplied so that innumerable colonies developed; ∞ means innumerable.

of one of these solutions and serum forms a good culture medium for the bacteria. Table II shows the same results as those set forth in Table I, but in these experiments rabbit serum and white-rat serum were tested on anthrax bacilli. Only the positive results are given in these tables. $(\text{NH}_4)_2\text{SO}_4$ and K_2SO_4 have relatively weak actions. Many other salts (KCl , LiCl , NaClO_3 , $\text{NaC}_3\text{H}_5\text{O}_3$, $\text{Na}_2\text{C}_4\text{H}_4\text{O}_6$, Na_2HPO_4 , Na_2CO_3 , NH_4Cl , $(\text{NH}_4)_2\text{CO}_3$, $(\text{NH}_4)_2\text{HPO}_4$, and urea) were experimented with, but as

TABLE II.

THE EFFECT OF SALTS ON LYSIS OF ANTHRAX BACILLI BY NORMAL RABBIT AND RAT SERUM.

RABBIT SERUM			WHITE RAT SERUM		
$\frac{m}{8}$ Solutions	At Once	4-5 Hours	$\frac{m}{8}$ Solutions	At Once	4-5 Hours
NaCl	93	0	NaCl	} 2,100	0
CaCl_2 0.4	110	2,000	CaCl_2 0.4		2,800
BaCl_2 0.4	100	30	BaCl_2 0.1		4,000
SrCl_2 0.4	115	95	SrCl_2 0.4		3,000
" 0.2	110	17	" 0.2		3,500
MgCl_2 0.4	90	170	MgCl_2 0.4		∞
K_2HPO_4 0.8	110	150	K_2HPO_4 0.4		6,000
" 0.4	100	140	" 0.2		6,000
$(\text{NH}_4)_2\text{SO}$ 0.4	115	150	$(\text{NH}_4)_2\text{SO}_4$ 0.8		8,000
" 0.2	110	110	" 0.5		8,000
K_2SO 0.8	140	160	K_2SO_4 0.4		35
" 0.4	90	44	" 0.2		60
MgSO_4 0.4	200	350	MgSO_4 0.8		9,000
" 0.2	210	500	" 0.5		10,000
$\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ 0.4	20	900	$\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ 0.4		3,000
" 0.2	20	800	" 0.2		3
$\text{Na}_2\text{C}_2\text{O}_4$ 0.4	128	140	$\text{Na}_2\text{C}_2\text{O}_4$ 0.4	} 1,800	150
" 0.2	150	0	" 0.2		30
$(\text{NH}_4)_2\text{C}_2\text{O}_4$ 0.4	80	300	$(\text{NH}_4)_2\text{C}_2\text{O}_4$ 0.4		3,000
" 0.2	130	150	" 0.2		4,500
K_4FeCn_6 0.4	120	125	K_4FeCn_6 0.4		1,500
" 0.2	110	18	" 0.2		11
$\text{K}_6\text{Fe}_2\text{Cn}_{12}$ 0.4	100	180	$\text{K}_6\text{Fe}_2\text{Cn}_{12}$ 0.4		1,200
" 0.1	120	120	" 0.2		90
$\text{NaHC}_4\text{H}_4\text{O}_6$ 0.05	180	600	$\text{NaHC}_4\text{H}_4\text{O}_6$ 0.05		4,000

NOTE.—Each tube contained 0.2 c.c. of serum + 0.1–0.8 c.c. $\frac{m}{8}$ solution of salt + 0.85 per cent. sodium chloride solution enough to make 1 c.c.

they had no marked effect on bacteriolysis, they were omitted from the tables.

The addition of these salts to the sera inhibits the bacteriolytic

action, but does not destroy that property of the serum, as is shown by the following experiment:

	At Once	3-4 Hrs.
0.2 c.c serum + 1 c.c. NaCl + B. typhosus	- - - 1,500	0
0.2 c.c serum + 0.6 c.c NaCl + 0.4 c.c SrCl_2 + B. typh.	- - 1,500	1,650
0.2 c.c serum + 4 c.c SrCl_2 + 0.4 c.c $\text{Na}_2\text{C}_2\text{O}_4$ + B. typh.	- - 1,450	0

A mixture of SrCl_2 and serum makes a good culture medium for typhoid bacilli, but if the SrCl_2 is precipitated out of solution by the addition of a proper quantity of $\text{Na}_2\text{C}_2\text{O}_4$, the serum again becomes bactericidal. This result may also be obtained with CaCl_2 , which may be precipitated with Na_2HPO_4 or $\text{Na}_2\text{C}_2\text{O}_4$, and also with $(\text{NH}_4)_2\text{C}_2\text{O}_4$ which may be precipitated with SrCl_2 or CaCl_2 .

Concentrated solutions of sodium chloride, as $\frac{m}{2}$ solution, also inhibit bacteriolysis, but the bacteriolytic power of the serum is again restored by diluting the mixture with distilled water.

These results harmonize very well with those of the similar experiments with hemolytic sera. The same solutions that suspend hemolysis suspend bacteriolysis, as a rule. While it is exceedingly difficult to determine the mechanism of suspension in the case of bacteriolysis, there is reason to believe from analogy that here, too, the complement is the point of attack.

We have been interested especially in some of Behring's early work on immunity to anthrax. Behring¹ investigated the causes of immunity to anthrax on the part of white rats, the serum of which is destructive of anthrax bacilli, but he found that neutralization by means of acids makes it a good culture medium for these organisms. The reduction of the alkalinity during life by the injection of oxalic acid also rendered the serum a good culture medium for anthrax bacilli. He made titrations which show that the serum of white rats is more alkaline than other sera which are not destructive of anthrax bacilli, and concluded from these observations that the anthracidal action of rat serum is due to alkalinity. This conclusion cannot stand, however, because the lytic action of rat serum, like that of other sera, depends upon interaction of amboceptor and complement; rat serum loses its anthracidal power by heating to 58°C . for thirty minutes, but, as its

¹ *Loc. cit.*

alkalinity is not reduced thereby, the bacteriolytic effect cannot be ascribed to the alkalinity alone. It seems more likely that the suspension of bacteriolysis noted by Behring on the addition to the serum of acids, especially oxalic acid, depended on neutralization of the complement.

Having found that so many salts inhibit bacteriolysis by serum, we now attempted to reduce the bacteriolytic power of the serum by injecting salts into animals during life. These experiments are however, still incomplete and unsatisfactory, because it is very difficult to inject enough of any of the salts without causing death of the animals. White rats were injected with 2 c.c. $\frac{m}{8}$ solution of $MgCl_2$, and then with anthrax, while one control was injected only with the $MgCl_2$ and another only with anthrax. This experiment was repeated several times, and the rats which had been injected with both $MgCl_2$ and anthrax died first. But the controls which received only anthrax also died, with the exception of one rat. The other controls were not sick. These experiments are unsatisfactory also because the rats at our disposal were less than a year old, and it is well known that young rats are more susceptible to infection with anthrax than old rats.

THE ACTION OF SALT SOLUTIONS UPON HEMOLYSIS BY NORMAL SERA.

In our experiments each tube contained 0.05–0.1 c.c. of serum and 0.2 c.c.–0.4 c.c. of $\frac{m}{8}$ salt solution, with sufficient 0.85 per cent. sodium chloride solution to make 1.0 c.c., and 1.0 c.c. of a freshly made 5 per cent. suspension (NaCl) of carefully washed corpuscles. In all cases the serum solution mixtures were placed at 36° C. for at least one-half hour, and never longer than two hours, before adding the corpuscles. The tubes were then placed at 36° C. for two hours, and subsequently in the ice-box for from sixteen to twenty-four hours. So far as possible, the different sets of experiments—for instance, those dealing with one kind of corpuscle or one kind of serum—were made at the same time with the same sera and the same corpuscular suspensions, all being fresh, so that the conditions were practically uniform, and the results consequently comparable, within certain limits. The reactions of all the mixtures were for the most part alkaline, a few appearing neutral to

litmus. The salts used for making the solutions were the purest obtainable, and the water used was first distilled three times over glass. Too much stress cannot be placed upon the necessity of the most careful control of the purity of the salts used in this kind of work. Contamination and alterations in the salt used in making the solutions may prove sources of grave error. In the course of these experiments some sodium fluoride, presumably of reliable make, was found to exercise marked antihemolytic action upon sera. This must have been due to some gross impurity. Unfortunately, it has not been possible to use the same solutions throughout the whole series of experiments. New solutions had to be made from time to time, but the salts used have always been the best obtainable. In order to record the results as accurately as possible, the amount of hemolysis has been put down in percentages determined by means of a colorimetric scale obtained by laking decreasing amounts of corpuscles in 2 c.c. of water, 100 per cent., the starting-point being formed by laking in 2 c.c. of water the corpuscles contained in 1 c.c. of the suspension used in the corresponding experiment; 90 per cent. would be 2 c.c. of water and 10 per cent. less corpuscles, etc.

In the case of certain sera—*e. g.*, dog and cat—considerable difficulty may be met in reading the results on account of cloudiness of the serum, apparently due to the presence in it of finely divided fat. In such cases, as well as in all cases requiring great accuracy, it is well to dilute with salt solution the mixtures as well as fluids composing the colorimetric scale up to several times the original quantities. Smaller differences in color, especially toward the high end of the scale, may now be determined with much greater accuracy. In the case of strongly agglutinative sera the rapid settlement of the corpuscles at the bottom of the tubes may interfere somewhat with the lytic action, which always requires some time for full development, especially when small quantities are used of but moderately active serum.

In order to obtain as accurate an idea as possible of the degree or strength of antihemolytic action of the salts studied in these experiments, the quantities of serum employed have in most cases barely been sufficient to cause complete laking of 1.0 c.c. of the

5 per cent. corpuscular suspension. In the case of sheep, rabbit, and swine sera, however, the hemolytic power of the quantities employed in the experiments fell far short of this standard.

Table III gives a general idea of the extent of our observations, so far as concerns the variety of sera and corpuscles used, and also the general nature of the results obtained with the salts to which it is desired to direct special attention at this time. We

TABLE III.

THE ACTION OF SALT SOLUTIONS UPON HEMOLYSIS BY VARIOUS SERA.

SOLUTIONS ($\frac{m}{s}$)	HUMAN CORPUSCLES + SERUM OF					RABBIT CORPUSCLES + SERUM OF									
	Goat	Sheep	Beef	Dog	Rab't	Goat	Sheep	Beef	Dog	Man	Horse	Cat	Chicken	Swine	
	0.1	0.15	0.1	0.1	0.1	0.1	0.15	0.1	0.1	0.1	0.2	0.1	0.2	0.1	
NaCl.....	80	25	80	60	30	100	100	100	80	80	100	80	100	20	
CaCl ₂	20	0	5	10	0	25	0	0	0	0	10	0	0	0	
BaCl ₂	0	0	0	0	0	10	0	0	0	0	0	0	20	0	
SrCl ₂	0	0	0	0	0	10	0	0	0	0	0	0	20	0	
MgCl ₂	25	0	15	0	0	20	20	10	0	10	10	50	35	0	
K ₂ SO ₄	10	0	30	15	30	85	10	40	10	45	0	35	100	5	
MgSO ₄	0	0	10	15	5	65	20	40	30	45	20	50	100	5	
(NH ₄) ₂ CO ₃	30	0	0	0	5	20	100	45	35	40	50	50	40	100	
NaHCO ₃	15	0	0	0	10	10	100	30	55	50	50	70	0	50	
Na ₂ C ₆ H ₅ O ₇	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Na ₂ C ₂ O ₄	0	0	0	0	0	10	0	5	0	0	0	0	5	0	
(NH ₄) ₂ C ₂ O ₄	0	0	0	0	0	30	5	12	0	0	0	0	0	5	
K ₄ Fe(Cn) ₆	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
K ₆ Fe ₂ (Cn) ₁₂	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

SOLUTIONS ($\frac{m}{s}$)	GUINEA-PIG CORP. + SERUM OF					CH'CK'N CORP. + SER. OF		HORSE CORP. + SERUM OF			W. RAT CORP. + SER. OF		DOG SERUM 0.1 + CORP. OF			
	Goat 0.1	Beef 0.1	Sheep 0.15	Swine 0.1	Dog 0.05	Goat 0.1	Dog 0.1	Goat 0.1	Man 0.15	Dog 1.1	Chicken 0.2	Dog 0.3	Beef	Sheep	Swine	Goat
NaCl.....	100	100	100	40	100	80	100	100	100	100	100	70	90	100	90	90
CaCl ₂	35	5	5	10	20	0	10	0	0	0	40	10	10	10	0	0
BaCl ₂	0	0	10	10	10	0	0	0	0	0	30	10	0	10	0	0
SrCl ₂	5	0	3	0	10	5	10	0	0	5	50	20	0	10	0	0
MgCl ₂	50	50	30	40	20	50	50	50	10	70	60	70	10	30	10	0
K ₂ SO ₄	50	80	10	40	90	40	100	50	40	100	75	0	40	30	100	90
MgSO ₄	60	100	50	40	90	40	100	40	10	10	90	20	40	30	100	30
(NH ₄) ₂ CO ₃	10	30	5	40	70	50	30	100	100	100	20	30	25	30	10	10
NaHCO ₃	10	20	20	20	60	50	10	100	100	100	50	10	20	20	10	0
Na ₂ C ₆ H ₅ O ₇	0	0	0	10	20	0	25	0	0	5	0	20	0	10	5	0
Na ₂ C ₂ O ₄	20	30	5	5	30	10	10	5	0	0	10	20	0	0	15	0
(NH ₄) ₂ C ₂ O ₄	20	20	25	10	10	20	10	0	30	20	20	30	5	20	20	0
K ₄ Fe(Cn) ₆	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
K ₆ Fe ₂ (Cn) ₁₂	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

NOTE.—In all the experiments tabulated in this article 0 means no recognizable hemolysis. It is probable that in all cases a small degree of lysis takes place.

have not tabulated the results of a number of experiments with KCl, NaFl, LiCl, NH₄Cl, NaI, NaBr, NaNO₂, NaNO₃, NaClO₃,

Na_2CO_3 , KCN, and several other substances (urea, oleate, lactate, etc.), because of the general absence in most instances of all, or at least of any marked, antilytic effect on the part of their solutions ($\frac{m}{8}$) in the proportions of 0.2 c.c. to 0.1 c.c. or so of serum. It is possible, of course, that some of the solutions may prove antilytic in certain cases and in larger quantities of $\frac{m}{8}$ solutions than those here used. In high concentrations— $\frac{m}{2}$ and $\frac{m}{3}$ —NaCl, KCl, as well as NaNO_3 , NaNO_2 , and $\text{KC}_2\text{H}_3\text{O}_2$, and probably also many others, prevent lysis by serum.¹ It is noteworthy that the corpuscles absorb amboceptors even in the concentrated solutions, observed also by Ehrlich and Sachs, and a few preliminary experiments indicate that the hemolytic power (as well as the bacteriolytic) of sera treated with such solutions is restored upon diluting the mixtures; evidently the antilytic action of the salts ($\frac{m}{8}$ solutions) in Table III depends on other factors than that of concentration.

There are, however, many salts other than those mentioned in Table III that will diminish or suspend lysis by serum in $\frac{m}{8}$ solutions; *e. g.*, tartrate (shown by Manwaring to be antilytic for goat serum, and by us to be antibacteriolytic), salicylate, benzoate, thiosulphate, and tungstate of sodium. These have not been included in a sufficient number of experiments to warrant further consideration at this time. Numerous experiments have been made with K_2HPO_4 , $(\text{NH}_4)_2\text{HPO}_4$, $(\text{NH}_4)_2\text{SO}_4$, KCNO and KCNS, but, on account of variability, the results are as yet not ripe for full discussion. CaCl_2 , BaCl_2 , SrCl_2 , $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, $\text{Na}_2\text{C}_2\text{O}_4$, $(\text{NH}_4)_2\text{C}_2\text{O}_4$, $\text{K}_4\text{Fe}(\text{CN})_6$, and $\text{K}_6\text{Fe}_2(\text{CN})_{12}$ constantly and completely, or nearly completely, suspend hemolysis by normal sera under the conditions peculiar to these experiments. It should be remembered that normal serum may vary in its hemolytic power, so that our results must not be accepted in too absolute a sense.

MgCl_2 , K_2SO_4 , and MgSO_4 have proved rather disappointing, because the earlier experiments with human serum and rabbit corpuscles showed them to possess decided antilytic properties, which they, however, have not maintained in the work with other

¹ See also von Lingelsheim's and Mark's experiments, *loc. cit.*

sera. It is possible that more concentrated (because older) solutions were used at first. Impurities may also have been present. These salts have also only a weak antibacteriolytic action.

INHIBITION BY SALT SOLUTIONS OF THE ACTIVATION BY GUINEA-PIG SERUM OF THE HEMOLYTIC AMBOCEPTORS IN COBRA VENOM.

Kyes¹ has shown that the hemolytic amboceptor of cobra venom is activated by lecithin, with which the amboceptor may form a crystallizable chemical compound—a discovery of the greatest significance to the study of lysins. Cobra venom is also activated by certain constituents of normal serum that in every way correspond to the complements in the usual sense of the word. The careful experiments of Kyes and Sachs² show conclusively that the complement in guinea-pig serum which activates cobra venom hemolytically is different and distinct from lecithin. For the purpose of studying the actions of antilytic salt solutions upon lecithin and guinea-pig serum in their rôle as activators of the hemolytic amboceptors of cobra venom, the following experiments were made (Table IV).

The recorded quantities of lecithin and guinea-pig serum were mixed with salt solutions, made up to 1 c.c. by means of sodium chloride solution, and placed in the incubator at 37.5° C. for two hours; at the end of this time the venom and corpuscles were added, the tubes returned to the incubator for two hours, and then placed in the ice-box over night. The experiment was repeated several times, with practically uniform results. In some of the mixtures containing salt solutions and lecithin there was a noticeable delay in the laking; *e. g.*, the CaCl_2 , BaCl_2 , MgSO_4 , NaHCO_3 , $\text{K}_4\text{Fe}(\text{CN})_6$, and $\text{K}_6\text{Fe}_2(\text{CN})_{12}$ tubes. Certain beef corpuscles are more susceptible to laking by the ammonium salts used, and by sodium bicarbonate, than others. In the experiments recorded in Table IV perfectly fresh corpuscles were used, and in this case only the $(\text{NH}_4)_2\text{CO}_3$ and NaHCO_3 solutions (0.5 c.c. + 0.5 c.c. NaCl sol. + 1 c.c. 5 per cent. suspension of corpuscles) caused any marked laking—about 40 per cent. Other experiments would have to be made in order to show to what

¹ *Berl. klin. Wechnschr.*, 1903, 40, p. 956.

² *Ibid.*, 1903, 40, p. 21.

TABLE IV.

ACTION OF SALT SOLUTIONS ON THE LYSIS OF BEEF CORPUSCLES BY COBRA VENOM ACTIVATED (a) BY LECITHIN (b) BY GUINEA-PIG SERUM.

SOLUTIONS ($\frac{m}{8}$)	SUSPENSION OF BEEF BLOOD (5 PER CENT.), 1 C.C., AND COBRA VENOM, 0.0002 C.C. +		WASHED, NON-HEMOLYZED, VENOM-SERUM COR- PUSCLES +	
	(a) Lecithin, 0.0001 c.c.	(b) Guinea-Pig Serum 0.2 c.c.	(a) Lecithin, 0.0001	(b) Guinea-Pig Serum, 0.4 c.c.
NaCl	100	100		
CaCl ₂ 0.5.....	100	5	+	+
BaCl ₂ 0.5.....	50	4	+	+
SrCl ₂ 0.5.....	100	10	+	+
MgCl ₂ 0.5.....	100	20	+	+
K ₂ HPO ₄ 0.5.....	100	100		
(NH ₄) ₂ HPO ₄ 0.5.....	100	100		
(NH ₄) ₂ SO ₄ 0.5.....	100	100		
K ₂ SO ₄ 0.5.....	100	70		
MgSO ₄ 0.5.....	100	10	+	+
(NH ₄) ₂ CO ₃ 0.5.....	100	100		
NaHCO ₃ 0.5.....	100	90		
Na ₃ C ₆ H ₅ O ₇ 0.5.....	20	0	+	+
Na ₂ C ₂ O ₄ 0.5.....	10	5	+	+
(NH ₄) ₂ C ₂ O ₄ 0.5.....	100	20	+	+
K ₄ Fe(Cn) ₆ 0.5.....	100	0	+	+
K ₆ Fe ₃ (Cn) ₁₂ 0.5.....	+	0		
KCNÖ 0.5.....	100	100		
KCNS 0.5.....	100	100		

*Color masked by the color of the salt solution.

degree, if any, these solutions prevent lysis by venom + guinea-pig serum or lecithin.

Both lecithin and guinea-pig serum cause hemolysis when added to the washed intact corpuscles in those cases in which the hemolytic action of venom + serum were suspended wholly or in part by salt solutions, as shown in Table IV. These corpuscles were washed twice in large quantities of NaCl solution and resuspended in 2 c.c. of the latter. This indicates that the corpuscles were venomized in the serum-salt-venom mixtures. Only minute traces of laking developed in the control experiments in which the corpuscles were treated similarly with serum-salt mixtures and venom, then washed, and resuspended without the addition of the lecithin or serum. In the case of the potassium ferricyanide experiment, however, the corpuscles underwent complete laking in the course of the washing.

These results support the conclusions of Kyes and Sachs that

lecithin and serum complements in the usual sense are different and distinct substances. The fact that sodium citrate and sodium oxalate greatly diminish lysis by venom and lecithin is not without interest. (It may be recalled that Kyes and Sachs found that guinea-pig serum contains a substance which inhibits activation of cobra venom by lecithin. The inhibitory effect is exercised by so small quantities of serum that the complement in the latter does not suffice to activate the venom.) But the results shown in Table IV also support the conclusion that the antilytic action of salt solutions is due to their effect upon the complement.

THE ANTILYTIC ACTION OF DISINTEGRATED TYPHOID BACILLI.

In view of the bactericidal effect of human serum on typhoid bacilli, the development of typhoid fever, in which the bacillemia of recent investigations must play an important rôle, appears at first sight somewhat puzzling, if we assume that the blood has the same action *in vivo* as the serum *in vitro*. The full discussion of this problem does not fall within the scope of this paper. The similar problem of anthrax has been studied recently, with interesting results, by Bail and Pettersson.¹

The possibility that the disintegration of typhoid bacilli might give rise to antilytic substances led to the following experiments:

A large excess of young typhoid bacilli was added to a quantity of human serum, incubated for eight hours, diluted with an equal volume of salt solution, and passed through a porcelain filter. This serum was now no longer bactericidal, but had decided antibactericidal properties, as is shown by the following experiment:

Filtrate	Human Serum	TYPHOID BACILLI	
		At Once	6 Hours
1 c.c.	0.1 c.c.	5,000	0
1 c.c.	0.05 c.c.	5,000	10,000
1 c.c.	0.025 c.c.	5,000	∞
NaCl Sol.			
1 c.c.	0.1 c.c.	5,000	0
1 c.c.	0.05 c.c.	5,000	0
1 c.c.	0.025 c.c.	5,000	0

¹ *Loc. cit.*

The objection might be raised that the difference in bacteriolysis in these two sets of tubes is due to the fact that the exhausted serum forms a good nutritive medium. This objection is rendered improbable, however, by the fact the exhausted serum has also antihemolytic properties in that it protects guinea-pig corpuscles against human serum.

The products of autolysis of typhoid bacilli also have antilytic properties. Following the method of Neisser and Shiga, twenty-four-hour growths of typhoid bacilli on glycerin agar slants were suspended in salt solution (10 c.c. for each tube), the suspension heated for one hour at 60° C., incubated at 36° C. for forty-eight hours, and passed through a porcelain filter. The colorless, transparent fluid thus obtained is neutral in reaction. In quantities of 1–0.1 c.c. to 0.1–0.2 c.c. of serum this fluid has been found to suspend lysis of sheep corpuscles by immune goat serum and of sensitized sheep corpuscles by normal goat serum; of rabbit corpuscles by human serum; of human, guinea-pig, and beef corpuscles by dog serum; and of human, guinea-pig, and beef corpuscles by sheep serum. Not all fluids of this kind have the same antilytic strength, and the lytic power of the different sera also varies considerably. Beef serum has not been inhibited by this fluid, heating of which to the boiling-point has not affected the antilytic action.

The antilytic action of the soluble products of autolysis of typhoid bacilli seems to be due to the effect upon the complement, because guinea-pig corpuscles are sensitized in dog serum mixture and sheep corpuscles in the immune goat serum mixture, as shown by prompt lysis after washing the corpuscles and adding the proper complements in small doses (0.05 normal guinea-pig serum in the first case and 0.005 normal guinea-pig serum or 0.05 normal goat serum in the second case).

Soluble products of autolysis of typhoid bacilli also hinder bacteriolysis, as shown by the following experiment:

	At Once	4 Hrs.
Autolytic fluid 1 c.c. + human serum + typhoid bacilli	3,600	1,500
NaCl solution 1 c.c. + human serum + typhoid bacilli	3,500	0

The results of these experiments may be taken to indicate that

in typhoid infections not only is complement used up, in the course of the bacteriolysis, which no doubt is going on, but that a certain amount is also neutralized by the soluble products of disintegration of typhoid bacilli.

Wilde¹ interpreted the removal of lytic properties of serum by dead bacteria as evidence against the amboceptor-complement nature of lysins. Closer analysis of his results might have shown, however, that the opposite conclusion would have been correct.²

THE MODE OF ACTION OF ANTILYTIC SALT SOLUTIONS AND THEIR RELATIONS TO THE COMPLEMENTS.

There can be no question that the antilytic salts now considered prevent lysis by virtue of their action on the complement. This must be so (1) because the corpuscles freely take up the intermediary bodies in the various non-lytic serum salt mixtures; (2) because precipitation of the inhibiting salt restores the lytic property of the complement; (3) because of the interesting quantitative relationships between complements and antilytic salt solutions.

1. Repeated experiments show that corpuscles become sensitized.³ *i. e.*, absorb intermediary body, in the non-lytic serum salt mixtures. The treated corpuscles were washed two or three times in sodium chloride solution, and then subjected to the action of pure complement. In all cases complete or marked lysis resulted promptly with minimum doses of complement, thus showing that the absence of lysis in the mixtures is not due to *Komplementoid-verstopfung* (Ehrlich and Sachs) of the amboceptor. In the controls of washed treated corpuscles suspended in sodium chloride solution without the addition of complement no laking takes place. (Ferrocyanide and ferricyanide of potassium seem to render guinea-pig corpuscles susceptible to laking under these circumstances.) It may be emphasized here that in no case has the treatment of sensitized corpuscles with antilytic solutions inter-

¹ *Loc. cit.*

² We have found since writing the above that Wright and Windsor (*Jour. of Hyg.*, 1902, 2, p. 385) observed that digestion of serum with typhoid or cholera cultures produces antibactericidal substances.

³ Throughout this article the word "sensitized" is used only in the sense here indicated, and not in the sense in which some French authors speak of "sensibilization."

ferred with lysis after careful washing and the addition of complement.

It has not been possible to go through the entire list of experiments in the manner just indicated, but it has been determined that absorption of amboceptor in non-lytic serum salt mixtures takes place in the case of human corpuscles treated with beef, sheep, and goat serum (washing and adding corresponding complements); of rabbit corpuscles treated with human serum and immune guinea-pig serum; of sheep corpuscles treated with immune goat serum, and of guinea-pig corpuscles treated with dog, beef, and horse serum. The guinea-pig corpuscles treated without hemolysis by dog and beef serum were laked, after washing, by adding 0.1 c.c. of normal guinea-pig serum, and those not laked by goat serum were laked after washing and adding 0.1 c.c. horse serum. These results, taken in connection with Manwaring's observations to the same effect, as regards dog corpuscles and immune goat serum, show that the salts suspend lysis by action on the complement, which is prevented from joining the amboceptor. That corpuscles absorb intermediary bodies in non-lytic mixtures of sera and salt solutions is shown also by simply adding to such mixtures containing intact corpuscles a surplus of pure complement, *e. g.*, normal guinea-pig serum, and normal goat serum to rabbit and sheep corpuscles treated respectively with immune guinea-pig serum and goat serum, which is followed by prompt laking, the anticipated result, if the conclusion that the antilytic salts act on complement is well founded.

2. Manwaring found that precipitation of inhibiting salts restores the lytic action of complement in goat serum. We have verified this fact for a number of sera, normal as well as immune, so far as inhibition by CaCl_2 , BaCl_2 , SrCl_2 , and $\text{Na}_2\text{C}_2\text{O}_4$ is concerned, but attempts to liberate the complements in ferro- and ferricyanide mixtures by precipitation with equivalent quantities of $\frac{m}{8}$ solutions of nickel and copper chloride met with failure. As already pointed out, the bacteriolytic action of serum is also restored by precipitation of the antisalt—a definite indication that here, too, the complement is the point of attack.

In some instances active hemolytic complement was liberated

from mixtures containing neutralizing doses of CaCl_2 , BaCl_2 , and SrCl_2 , by precipitation of the latter several days after the complement had disappeared from the control specimens of untreated serum. Here, one might say, it would seem as if the salt solutions rendered the complement more resistant to the influences that lead to its so-called spontaneous degeneration; or that the ion or salt-complement compound is more stable than the form in which complement naturally occurs in serum.

3. In case the solutions prevented lysis by direct action upon the bodies of the corpuscles (or the bacteria), one would hardly expect any such accurate quantitative relationship between the solutions and the complements as is the actual case. The protection against lysis by a given quantity of $\frac{m}{8}$ solution is quickly overcome when the amount of serum or complement exceeds a certain definite limit, indicating that there is nothing in the way of free complement directly uniting with the sensitized corpuscle and causing its lysis. While 0.2 c.c. of CaCl_2 or of $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ protect guinea-pig corpuscles against lysis by 0.1 c.c. of goat serum, prompt laking occurs if to this mixture is added 0.05 c.c. of dog serum. The same result is obtained if we reverse the sera, indicating again that the corpuscles are as susceptible as ever to active serum. Similar experiments with sheep corpuscles sensitized with heated immune goat serum and the complement in normal goat serum and guinea-pig serum give the same result.

Here may be mentioned again that the activation of cobra venom by guinea-pig serum is prevented by a number of salt solutions, which, however, have no such action on lecithin—a phenomenon most readily explained as due to the direct action of the salts on the guinea-pig complement.

Manwaring also finds that the amount of salt necessary to prevent lysis varies directly with the amount of complement in the serum used. Tables V and VI record more recent experiments with other sera, normal as well as immune, the results of which show that decreasing amounts of antilytic solutions give increasing lysis with the same quantity of complement or serum; that increasing quantities of complement give increasing lysis with the same amount of salt solution; and finally that multiple pro-

portions of the amount of complement and salt in a given mixture maintain about the same lytic value. The quantitative relationships are of such a character that there seems to be good reason to believe that in their mutual action these substances follow demonstrable physico-chemical laws.

When added to sera, some salt solutions may form by disso-

TABLE V.

QUANTITATIVE RELATIONS BETWEEN CERTAIN ANTILYTIC SOLUTIONS AND DOG SERUM.

DOG SERUM AND GUINEA-PIG CORPUSCLES.

CaCl ₂			NaHCO ₃			Na ₃ C ₆ H ₅ O ₇			Na ₂ C ₂ O ₄			K ₄ Fe(CN) ₆		
AMOUNT OF			AMOUNT OF			AMOUNT OF			AMOUNT OF			AMOUNT OF		
Sol.	Com.	Lysis	Sol.	Ser.	Lysis	Sol.	Ser.	Lysis	Sol.	Ser.	Lysis	Sol.	Ser.	Lysis
0.24	0.05	10	0.24	0.05	20	0.24	0.05	15	0.24	0.05	0	0.15	0.05	15
0.21	0.05	15	0.21	0.05	20	0.21	0.05	25	0.21	0.05	21	0.12	0.05	30
0.18	0.05	20	0.18	0.05	28	0.18	0.05	30	0.18	0.05	35	0.09	0.05	40
0.15	0.05	28	0.15	0.05	35	0.15	0.05	42	0.15	0.05	43	0.06	0.05	55
0.12	0.05	40	0.12	0.05	50	0.12	0.05	48	0.12	0.05	50	0.03	0.05	80
0.18	0.07	30	0.18	0.07	40	0.21	0.07	50	0.24	0.07	35	0.12	0.07	60
0.18	0.09	60	0.18	0.09	70	0.21	0.09	70	0.24	0.09	50	0.12	0.09	70
0.36	0.1	15	0.36	0.1	25	0.42	0.1	20	0.48	0.1	5	0.24	0.1	5
0.72	0.2	5	0.72	0.2	35	0.84	0.2	40	0.96	0.2	5	0.48	0.2	5

DOG SERUM AND SHEEP CORPUSCLES.

CaCl ₂			NaHCO ₂			Na ₃ C ₆ H ₅ O ₇			Na ₂ C ₂ O ₄			K ₄ Fe(CN) ₆		
AMOUNT OF			AMOUNT OF			AMOUNT OF			AMOUNT OF			AMOUNT OF		
Sol.	Ser.	Lysis	Sol.	Ser.	Lysis	Sol.	Ser.	Lysis	Sol.	Ser.	Lysis	Sol.	Ser.	Lysis
0.24	0.05	10	0.24	0.05	15	0.24	0.05	25	0.15	0.05	10	0.15	0.05	16
0.21	0.05	10	0.21	0.05	18	0.21	0.05	27	0.12	0.05	30	0.12	0.05	30
0.18	0.05	15	0.18	0.05	30	0.18	0.05	30	0.09	0.05	40	0.09	0.05	38
0.15	0.05	20	0.15	0.05	36	0.15	0.05	32	0.06	0.05	45	0.06	0.05	40
0.12	0.05	25	0.12	0.05	40	0.12	0.05	35	0.03	0.05	55	0.03	0.05	50
0.18	0.07	20	0.28	0.07	15	0.21	0.07	20	0.24	0.07	15			
0.18	0.09	30	0.28	0.09	30	0.21	0.09	30	0.24	0.09	25			
0.36	0.1	25	0.36	0.1	5	0.42	0.1	0	0.48	0.1	0			
0.72	0.2	5	0.72	0.2	5	0.84	0.2	0	0.96	0.2	0			

ciation substances in themselves lytic for *certain* corpuscles. Here some laking results in spite of antilytic action of the salt upon the serum. Our experiments show that in certain instances an excess of salt solutions, especially of those containing NH₃ and OH ions, above that necessary to prevent hemolysis undoubtedly causes considerable laking of some corpuscles. This is especially true of carbonate and oxalate of ammonium, and to a less degree of bicarbonate of sodium. Thus 0.1 c.c. of $\frac{m}{8}$ solution of ammo-

mium carbonate prevents or greatly reduces lysis of horse corpuscles by 0.1 c.c. of goat serum, and of horse and goat corpuscles by 0.1 c.c. of dog serum; but if the quantity of solution is increased, marked laking may result (Table VII). Similar illustrations are furnished also by rabbit corpuscles and sheep corpuscles. In our experiments the fact, emphasized by Arrhenius and Madsen, that the corpuscles bind a certain amount of NH_3 before

TABLE VI.

QUANTITATIVE RELATIONS BETWEEN CERTAIN ANTILYTIC SOLUTIONS AND COMPLEMENT.

SENSITIZED SHEEP CORPUSCLES + NORMAL GOAT SERUM.

CaCl ₂			BaCl ₂			NaHCO ₃			Na ₂ C ₆ H ₅ O ₇			Na ₂ C ₂ O ₄		
AMOUNT OF			AMOUNT OF			AMOUNT OF			AMOUNT OF			AMOUNT OF		
Sol.	Com.	Lysis	Sol.	Com.	Lysis	Sol.	Com.	Lysis	Sol.	Com.	Lysis	Sol.	Com.	Lysis
0.3	0.1	10	0.3	0.1	10	0.3	0.1	20	0.3	0.1	5	0.3	0.1	20
0.25	0.1	22	0.25	0.1	20	0.25	0.1	20	0.25	0.1	5	0.25	0.1	30
0.2	0.1	50	0.2	0.1	40	0.2	0.1	20	0.2	0.1	5	0.2	0.1	50
0.15	0.1	100	0.15	0.1	52	0.15	0.1	28	0.15	0.1	30	0.15	0.1	65
0.1	0.1	100	0.1	0.1	100	0.1	0.1	37	0.1	0.1	50	0.1	0.1	100
						0.1	0.05	20	0.1	0.05	10			
						0.2	0.1	20	0.2	0.1	10			

SENSITIZED SHEEP CORPUSCLES + NORMAL GUINEA-PIG SERUM.

CaCl ₂			NaHCO ₃			Na ₂ C ₆ H ₅ O ₇			Na ₂ C ₂ O ₄			K ₄ Fe(CN) ₆		
AMOUNT OF			AMOUNT OF			AMOUNT OF			AMOUNT OF			AMOUNT OF		
Sol.	Com.	Lysis	Sol.	Com.	Lysis	Sol.	Com.	Lysis	Sol.	Com.	Lysis	Sol.	Com.	Lysis
0.15	0.005	15	0.15	0.005	20	0.15	0.005	20	0.15	0.005	30	0.06	0.005	15
0.12	0.005	25	0.12	0.005	35	0.12	0.005	25	0.12	0.005	40	0.04	0.005	25
0.09	0.005	30	0.09	0.005	50	0.09	0.005	30	0.09	0.005	60	0.02	0.005	35
0.06	0.005	40	0.06	0.005	100	0.06	0.005	35	0.06	0.005	100	0.06	0.0075	30
0.15	0.0075	55	0.12	0.0075	100	0.03	0.005	40	0.12	0.0075	100	0.06	0.01	38
0.15	0.01	40	0.12	0.01	100	0.12	0.0075	30	0.12	0.01	100	0.15	0.0075	5
0.45	0.0075	15	0.45	0.0075	20	0.12	0.01	35	0.45	0.0075	20	0.2	0.01	5
0.6	0.01	15	0.6	0.01	20	0.45	0.0075	20	0.6	0.01	20			
						0.6	0.01	20						

NOTE.—Special mention must be made of agglutination as a source of error, especially in experiments with immune serum. The tendency to agglutination not only makes it difficult to secure an equal distribution of the corpuscles in the suspension, but the rapid settlement of the corpuscles may interfere more or less with lysis.

laking occurs, is noticeable. It seems also that serum—heated as well as unheated—may interfere with the laking by ammonia. It is quite likely that the extent to which ammonia may be bound by different corpuscles and sera is subject to considerable variation, and that this may be a factor in the variability of the results

in the antilytic experiments with many ammonium salts. CaCl_2 solution is also lytic for some corpuscles, unless bound or neutralized by substances in the serum (complement?), as observed also by Manwaring—a fact that hardly would harmonize with the idea that CaCl_2 prevented lysis by direct action on the corpuscles.

The practically uniform antihemolytic (and antibacteriolytic) actions of CaCl_2 , BaCl_2 , SrCl_2 , $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, $\text{Na}_2\text{C}_2\text{O}_7$, $(\text{NH}_4)_2\text{C}_2\text{O}_4$, $\text{K}_4\text{Fe}(\text{CN})_6$, and $\text{K}_6\text{Fe}_2(\text{CN})_{12}$, upon all sera examined

TABLE VII.

LYSIS BY EXCESS OF ANTILYTIC DOSE OF $(\text{NH}_4)_2\text{CO}_3$ AND $(\text{NH}_4)_2\text{C}_2\text{O}_4$.

SOLUTIONS ($\frac{m}{8}$)	SHEEP CORPUSCLES +		GOAT CORP. +	HORSE CORPUSCLES +		GUINEA- PIG CORP. +	RABBIT CORP. +
	Dog Ser., 0.05	Immune Goat Ser., 0.1	Dog Ser., 0.1	Dog Ser., 0.1	Goat Ser., 0.1	Dog Ser., 0.05	Immune Guinea- Pig Ser., 0.05
NaCl	100	100	100	100	100	100	100
$(\text{NH}_4)_2\text{CO}_3$ 0.5	35	20	20	100	60	75	100
“ 0.4	25	10	0	85	40	70	100
“ 0.3	5	0	0	30	30	20	70
“ 0.2	5	0	0	20	20	20	40
“ 0.1	25	5	10	30	0	30	0
“ 0.05	50	30	30	30	5	70	20
$(\text{NH}_4)_2\text{C}_2\text{O}_4$ 0.5	35	40	60				
“ 0.4	25	40	45				
“ 0.3	20	20	10				
“ 0.2	20	5	0				
“ 0.1	35	40	0				
“ 0.05	100	80	80				

speaks strongly in favor of great similarity of nature and structure of various complements in various sera and in the same serum. Whether it is possible on the strength of the data at hand in regard to certain salts with variable antihemolytic action (see Table III) to make out any real quantitative differences in complements is very doubtful, because our experiments with normal sera have been made on the same general plan, without any attempts to determine the exact lytic dose of complement for the amount of amboceptor in each case.

Were this done, and the antilytic effect of varying quantities of the solutions then determined, a much truer estimate would be

possible of their real value. In other words, accurate quantitative experiments, which can be made best with immune sera, are necessary to determine the exact antilytic value of a salt. Inasmuch as many sera contain more than one complement, and inasmuch as the antisalts, as a rule, seem to act on all complements in a serum, it becomes clear that, in order to determine with utmost exactness the precise antilytic dose of a solution, it would be necessary to have a serum with but one complement.

No doubt the relative amounts of amboceptor and complement vary in the sera of different animals, and even in the same animal at different times. Now von Dungern, Gruber, and especially Morgenroth and Sachs,¹ show that in the presence of large amounts of amboceptors relatively small amounts of complements suffice for lysis. Hence in normal sera with high complement content enough free complement may remain in some serum salt mixtures to cause more or less laking, depending on the amount of amboceptor, and thus variation in the relative amount of amboceptor and complement may help to account for variations in the antilytic action of salts. In the course of the experiments with sensitized corpuscles and complement (Table VI), Morgenroth and Sachs's observations on the effect of larger doses of amboceptor in diminishing the amount of complement necessary for lysis could be confirmed frequently. Owing to this relationship between complement and amboceptor, it is difficult to obtain uniform results in experiments with antilytic salts and immune sera.

Of the many other factors that would have to be considered in any attempt to discuss more fully the variations in the antilytic action of some salts we would mention: the conditions that influence the dissociation of the salts; the variations in the susceptibility of corpuscles to various forms of laking; in the case of the ammonium salts, the variations in the amounts of lytically active ammonia set free; and variations in the relative amounts of different complements in a given serum, as well as in the affinity for complement on the part of amboceptors.

As already pointed out, the interesting fact that complements in general are neutralized by certain salts indicates a close rela-

¹ *Berl. klin. Wchnschr.*, 1902, 39, pp. 517.

tionship in the chemico-physical nature of complements. Their ready response to this action of salts is in marked contrast to the behavior of the intermediary bodies, which appear wholly unaffected by the same salts in the quantities necessary to counteract the complements. Perhaps this important point of distinction may prove of service in the further study of these bodies.

At this time we do not wish to enter upon any extended discussion of the interesting, but difficult, problem of the exact mode of neutralization of complements by certain salts—whether the function of the atomic weight, the valency, the nature of the electrical charge,¹ or solution tension of the ions.²

CONCLUSIONS.

In small doses of $\frac{m}{8}$ solutions many salts prevent lysis of red corpuscles and of bacteria by various sera. The same salts inhibit the activation of cobra venom by guinea-pig serum.

The soluble substances of disintegrated typhoid bacilli have antibacteriolytic and antihemolytic properties.

The antilytic salts here considered prevent hemolysis, and probably also bacteriolysis, by virtue of their action on the complements, (1) because in non-lytic serum salt mixtures corpuscles freely take up amboceptors whose complementophile groups remain free; (2) because precipitation of antisalts restores lytic property of complement; (3) because of the quantitative relationship between complements and antisalts.

It is probable that in their reactions complements and antilytic salt solutions follow simple physico-chemical laws.

¹The following extract from one of LOEB's articles (*Popular Science Monthly*, 1902, 61, p. 80) may be given as representing his conception of the mode of physiological action of electrically charged molecules: "The fact that ions may act toxically through their electrical charges, and that ions with the opposite charge may act antitoxically, may open a new and very fertile field for pathology and therapeutics. . . . Two years ago I pointed out that we must realize the existence of physiologically balanced salt solutions, that means salt solutions in which the ions are so combined that the toxic effects of the one are counteracted by the antitoxic effect of some other ion. Any disturbance in the right proportions of monovalent ions and ions of higher valency must lead to more or less pronounced modifications of the life phenomena." In connection with this it is interesting to note that VON BEHRING (*Beiträge zur exp. Therapie*, 1904) attempts to explain the neutralization of tetanus toxin by antitoxin by the assumption that + energy is neutralized by - energy, without there necessarily being any chemical union of the antitoxin with the toxin.

²A. P. MATHEWS, *Amer. Jour. of Physiol.*, 1904, 10, p. 290.

That complements generally are neutralized by the same salts indicates a close relationship of their chemico-physical properties.

So far as their functions in lysis are concerned, amboceptors appear to remain unaffected by antilytic salts in quantities sufficient to neutralize the complements.

The susceptibility of complements to the influence of various non-specific anticomplements—*e. g.*, salts, glycogen (Wendelstadt), products of disintegrated typhoid bacilli—suggests that neutralization of complements may play an important rôle in certain infections.

ON THE LEUCOCYTOSIS IN SCARLET FEVER.*

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In the following observations an attempt has been made to verify the observations of previous writers, and further to note the amount of leucocytosis in different stages of scarlet fever, in different grades of severity of the disease, and also the effect of the various complications and sequelæ on the white blood count. Though there are changes in the various elements of the blood in scarlet fever, erythrocytes as well as leucocytes, it is to the condition of the white cells that I shall confine myself.

Pick, Halla, Rieder, and Kotschetkoff were among the first to report their observations in blood analysis of scarlet fever patients, and all were agreed on the constant presence of a leucocytosis. Their actual figures as to the intensity of the leucocytosis vary considerably, due no doubt to the varying intensity of the disease.

Kotschetkoff found, after examining the blood of twenty scarlatina patients, that the leucocytosis was directly proportionate to the severity of the cases, the variation lying between a minimum of 10,000 and a maximum of 40,000. The highest leucocytosis (40,000) he obtained in rapidly fatal cases. He found an early leucocytosis in all, reaching its height on the second or third day, then gradually decreasing to normal after six weeks. From his observations he concluded that neither temperature, lymphadenitis, otitis, nor nephritis had any effect on the number of leucocytes. In his cases the *überreiffen Formen*—the neutrophiles and eosinophiles—were increased in number to 85–98 per cent., but those having 95 per cent. or more of neutrophiles and eosinophiles ended fatally. These cells were found in greatest number on the second day of the rash, from which time on there was a daily decline to normal. After returning to normal, further complications had no influence on the count. In fatal cases the eosinophiles and neutrophiles remained at their maximum. Kotschetkoff found that the

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eosinophiles were increased, except in fatal cases, from the third day on till the third week, to as much as 15 per cent.

Zappert, from his studies on eosinophiles, concludes that in fevers, during the febrile intoxication, there is a fall in the eosinophiles. After the disappearance of the fever, the eosinophiles gradually ascend to normal. In scarlatina he agrees with Kotschetkoff in the early diminution of the eosinophiles, with a subsequent rise, reaching the maximum at the end of the third week.

Of the more recent writers on the subject, Sacquépée found that, in older patients, twenty to twenty-two years of age, the eosinophiles were increased early in the disease (fourth or fifth day), and the increase persisted for three or four weeks. The apparent increase in the mononuclears, he holds, is due to a more rapid decrease in number on the part of the polynuclears. Cervical adenitis and parotiditis, as complications of scarlatina, both show more or less leucocytosis. In fatal cases of scarlatina he observed a hyperleucocytosis with "abnormal" forms, which he found in none but malignant cases. The polynuclear cells, showing a decided acidophile nature, he regarded as degenerated eosinophiles.

Reckzeh found a maximum leucocytosis of 41,000, on the fourth to the ninth day. The decline in the number of leucocytes, he states, is gradual, and reaches its normal toward the third week. He found that adenitis, joint affections, and endocarditis each causes an increased leucocytosis. On the contrary, nephritis is followed by a decrease in the number of white cells. The lymphocytes are fewer at the onset, but later increase as the polynuclears decrease. The eosinophiles in his series varied from 1 to 12.5 per cent., reaching the maximum about the eighth day. He believes that the unknown scarlatinal toxin stimulates the blood-producing organs (as regards the leucocytes) to a greater activity, with a consequent increased white cell count.

Reckzeh gives a very full tabulated statement of the successive blood counts throughout the course of each of his ten cases, often as many as a dozen, and extending from the second or third day of the fever to the end of the fifth or sixth week. What appears to me not a little remarkable is that, in the majority of these

counts, he reports either a low count or a total absence of eosinophiles. This he does not refer to in his summing up, but, on the contrary, lays stress upon the eosinophilia. Judging from my observations, his "0" in many cases means either that he has failed to count the eosinophiles, or else that he has regarded the faintly stained, coarsely granular oxyphile cells among the neutrophiles. Reference to the plate accompanying this paper will, I think, establish that these are truly to be classed as eosinophiles proper.

Rille* observed that in two moderate cases of scarlatina the eosinophiles were moderate in number, though there was a leucocytosis varying from 8,800 to 18,200. In a fatal case he found the eosinophiles increased 5.3 to 7.77, with a leucocytosis of from 15,000 to 20,000.

Plantenga, comparing the results of his examinations of measles and *Rötheln* with those of scarlatina, concluded that a diagnostic point could be made of an early increase in the polynuclears in the two first-mentioned diseases. Reference to my tables does not support this view.

Mackie found in examining the blood of scarlet fever patients that the leucocytosis was marked in mild or moderate cases, while in unfavorable cases the leucocytosis was less marked or even absent.

While writing this article, after the completion of my observations, my attention was drawn by Dr. Hektoen to a singularly full and able paper on the subject by J. M. Bowie. Bowie examined many more cases than are taken up here, but I was very much struck with the similarity of his observations with those here given. What would appear to be discrepancies in our results I think can be explained by two facts: one, the constant variation in the intensity and nature of scarlatinal epidemics, and the other, our different classification of the varieties of the disease. No one will deny the great variation in the intensity and sequelæ to which scarlatinal epidemics are subject, which necessarily must also affect the leucocytosis. Bowie divides his cases into scarlatina simplex, scarlatina anginosa, and fatal cases. In the first group he considers only the mild, I presume uncomplicated, cases. In the second group he places all moderately severe

* Cited by Reckzeh.

cases, with and without complications. In my series, those spoken of as moderately severe I divide into two classes—the pure uncomplicated cases, and those with complications of adenitis, otitis, joint affections, and so on. Bowie also divides his fatal cases into two groups—"those that never recover from the primary shock of the fever, and those that make a bold bid for life, but die eventually from exhaustion and toxemia." Two of the cases to be considered later come under the first heading; a third, under the second. I shall take up his paper more fully in the summary, and compare the results with the present observations.

Remembering the frequent discovery of streptococcal complications in this disease, it becomes worth while to note the observations made regarding the variations in the leucocytes in septic, and more particularly in streptococcal, disorders.

Cabot has tabulated a series of cases of septicemia, and found that forty-two out of fifty-six gave a leucocytosis varying from 9,000 to 77,500. The leucocytosis was of the polymorphonuclear type, with a disappearance of the eosinophiles and a diminution of the lymphocytes. He further reports the examination of three cases in which the streptococcus was isolated, and showing a varying leucocytosis of from 24,800 to 46,000.

Rieder notes in a fatal case of sepsis that the eosinophiles are wholly absent.

Residence in an infectious disease hospital (the Ottawa Isolation Hospital) gave me an opportunity of examining a series of fourteen cases of undoubted scarlatina. Only those cases were utilized in which the clinical history and symptoms indicated the unquestionable nature of the disease. As all the cases are from hospital practice, examinations were not obtained earlier than twelve hours after the onset; hence no results can be given as to the leucocytosis appearing during the incubation period.

The ages of the patients ranged from two and a half to fourteen years. The examination consisted in the estimation of the white cells per cubic millimeter, and the preparation of stained slides for the estimation of the relative count, and the minute detail of the cells.

The blood was obtained in the usual way from the lobe of the ear. The examinations were made late in the morning, usually just before noon, to obtain counts as nearly as possible under similar conditions, and eliminate the leucocytosis following digestion. The examination was made with a Thoma-Zeiss hemocytometer, using a 3 per cent. acetic acid solution, tinged with gentian violet, as a diluting medium. Three successive counts were made of the complete 400 squares each, making a total of 1,200 squares.

The films were made on coverslips and fixed in a mixture of equal parts of alcohol and ether, being stained later by the Nocht-Romanowsky method. For the determination of the relative numbers of white cells present at least 800 cells were counted. In studying the more minute structure of the cells a one-twelfth oil immersion lens and No. 5 ocular were used (Reichert).

As normally there is a considerably higher count of white cells in children than in adults, a leucocytosis was considered only in the presence of 10,000 or over per cubic millimeter. The normal adult differential count may be considered as,

	Per cent.
Lymphocytes - - - - -	22-25
Polymorphonuclear neutrophils - - -	62-70
Eosinophiles - - - - -	0.5-4

In infancy and childhood the percentage of lymphocytes is higher (40-60 per cent.), and the polymorphonuclears are only 28-40 per cent. (Cabot).

The cases examined in these observations were: two cases of very mild scarlatina, nine of moderate severity, and three of malignant scarlatina, two of which died within three days after the onset. Among the cases of moderate severity were also those developing adenitis, otitis media, nephritis, and arthritis. In two patients, counts were obtained before and after the administration of antistreptococcic serum.

MILD SCARLATINA.

The diagnosis in these two cases rested mainly on the mode of onset, the presence of other cases of scarlet fever in the same family, and the subsequent desquamation.

In the very mild type of scarlet fever the white cells showed little or no change in number or relative count. This, then, resembles the condition found in pneumonia, where we also meet with a normal, or even subnormal, count during a mild attack. The highest count obtained in these two cases was 12,780 on the second day, while the lowest was 7,400. The differential counts were normal. The conditions here also bear a close resemblance to those seen in measles, where the count is always low.

Case I, female, <i>æt.</i> 6—	2d day	-	-	-	12,780
	9th day	-	-	-	9,228
	11th day	-	-	-	10,740
	28th day	-	-	-	11,680
Case II, female, <i>æt.</i> 2—	2d day	-	-	-	9,440
	12th day	-	-	-	7,400

In each of these cases the rash consisted of a diffuse blush over the body, which disappeared by the fourth day; the sore throat was very slight; and desquamation was of the fine branny character. In neither case were there any complications during convalescence.

SCARLATINA OF MODERATE SEVERITY.

This type of the disease presents to us the most interesting series, for in it we meet with some running an uncomplicated course of scarlet fever, while others develop adenitis, otitis media, and so on, thus giving us several classifications to be considered.

During the first week we may consider all the cases of this type of the disease under the same heading, as up to this time no complications have arisen.

Viewing the leucocytosis as it occurs on the different days, the results are as follows:

Day of Disease	No. of Cases Examined	Average Count
2d - - - - -	1	18,000
3d - - - - -	5	32,260
4th - - - - -	3	33,185
5th - - - - -	2	29,505
6th - - - - -	1	30,670
8th - - - - -	2	23,060

The highest individual count obtained in the above series was 78,133 on the third day of the disease. No reason could be assigned for this high count, as the leucocytes numbered only

28,730 on the fifth day, while the case ran its further course of moderate severity with no complications. The lowest count during the first week was 11,370.

In the above series, it will be seen, the leucocytosis ascends till the fourth day—which just about represents the course of the intensity of the scarlatinal rash.

None of the patients had a temperature above 103.2° , and the highest temperature was usually on the second or third day. The curve of the leucocytes does not follow the temperature curve. While we may note a sudden drop in the fever, this does not denote a consequent fall in the number of leucocytes, which gradually decrease in number till the fourth or fifth week.

From the eighth day on, the cases must be divided under several headings as complications arise:

Pure uncomplicated cases.—Of the uncomplicated cases of moderate scarlatina in the series there were five, in which the counts resulted as follows:

Day of Disease	No. of Cases Examined	Average Count
15th - - - - -	3	15,585
21st - - - - -	3	13,260
25th - - - - -	1	15,270
29th - - - - -	2	12,890
34th - - - - -	1	10,900

The tabulation shows a gradual decrease in the leucocytosis. During this time (from the end of the second week on) the temperature runs a normal course and desquamation is advancing. Desquamation of various intensity showed no change in the white-cell count.

Complicated cases.—One case, complicated with *adenitis* and *otitis media* (suppurative) during the third week, gave an increase in the leucocytosis. The discharge from the ear contained streptococci, staphylococci, and some large bacilli which were not determined.

Day of Disease	Count
15th - -	14,720
18th - -	Onset of adenitis and otitis media
19th - -	18,460
25th - -	16,250
32d - -	13,630

Two cases, complicated with *varicella*, gave an increased leu-

cocytosis at the onset of the second disease (the rise in temperature being the first symptom) and before the appearance of the vesicles. The first case occurred on the sixteenth day, with a consequent leucocytosis of 20,470—a rise of nearly 7,000 over an examination three days previously. The second case developed varicella nineteen days after the onset of scarlatina, and showed a leucocytosis of 18,060 on the day previous to the appearance of the vesicles. Forty-eight hours after the appearance of the vesicles, when these were becoming opalescent, while others were dying, the white-cell count amounted to 16,632.

One patient developed a severe *arthritis* on the forty-fourth day after the onset of scarlatina. The ankles, wrists, and knees were particularly affected, while the temperature rapidly rose to 104 and 105°. The leucocyte count up to this time had been that of moderate severity, the highest count being 26,720. The count on the thirtieth day was 14,056; on the forty-fifth day, the day following the onset of the arthritis, this was increased to 32,740. The temperature remained persistently high for some weeks, returning to normal at the end of the ninth week. On the fifty-sixth day the leucocyte count was 18,900, and at the end of the ninth week this fell to 14,348. The patient left the hospital at the end of the tenth week, and although the temperature remained about normal, the arthritis persisted for some time.

MODERATELY SEVERE CASES—DIFFERENTIAL COUNT.

Now, considering the differential count in cases of moderate severity, the following results were obtained:

Period of Disease	No. of Cases	Total No. of Leucocytes Averaged	Maximum No. in Any Case	Minimum No. in Any Case	AVERAGE PERCENTAGE OF		
					Poly-morphonuclears	Lymphocytes	Eosinophiles
2d-4th day.....	5	25,130	78,133	11,370	89.1	9.8	1.1
4th-10th day.....	5	29,080	48,270	16,400	85.6	10.7	3.7
10th-16th day.....	4	17,100	22,500	14,940	79.6	13.6	6.8
16th-22d day.....	4	13,080	23,060	9,300	69.2	14.5	16.3
22d-31st day.....	4	14,230	18,240	9,870	58.5	30.9	10.6
35th-42d day.....	3	11,400	13,500	8,900	59.3	47	0.5-4
Normal.....		9,500-10,500			30-50	40-60	0.5-4

The highest eosinophile count during the first week was 4.9 per cent. and the lowest 0.41. The highest polymorphonuclear count during the first week was obtained on the second day of the disease, the count reaching 96.7 per cent. of all cells. The lymphocytes were reduced in this case to 2.89 per cent.

In the early stages of the disease the polymorphonuclear cells show very poor staining qualities, the nuclei being indistinctly outlined. The chromatin films are much broken up and stain unequally. The fine granules outside the nucleus present a hazy purple appearance.

During the second week the highest individual eosinophile count was obtained, on the thirteenth day, there being 19.3 per cent. of these blood elements present. The coarse oxyphile cells stain very intensely; the granules are larger and are packed closely in the cells. The cells seem overburdened with the acidophile granules, and are readily broken in making blood films, in which case the field about the burst corpuscle shows many of these granules extra-cellular and well stained.

Toward the end of the second week the polymorphonuclear leucocytes also change in character. The fine oxyphile granules are now somewhat coarser and take the eosin stain more actively. These cells show their oxyphile character in all grades, from the faintly staining pink cells, whose individual granules can be distinguished, to those that show such intense and coarse staining that they closely resemble the true eosinophile leucocytes. This acid-staining characteristic was noted particularly in the severer cases which had passed the crisis.

From the end of the second week there was a constant decline in the proportion of the polymorphonuclears, while the lymphocytes increased. A lymphocytosis during convalescence was found only in a few instances, the highest proportion being 52.3 per cent. on the thirty-first day of the disease.

The eosinophiles run a course quite different from that of the other leucocytes. During the first few days the count is low, and even at times from day to day decreases, up to the fifth or sixth day, their number from this time on rapidly increasing, till a maximum is reached (in my series of cases) between the four-

teenth and twenty-first days. This initial decline with a secondary rise fairly accurately followed, inversely, the temperature curve, which in turn denotes the existing toxemia. Hence it would seem that during the time of the extensive elaboration of scarlatinal toxins in the body there is a low eosinophile count. From the end of the third week, in nearly all cases, the eosinophiles gradually decrease during convalescence. Slight exacerbations of temperature had no effect on the proportion of the coarse oxyphiles.

The cases complicated with adenitis and otitis media showed an increase mainly in the polymorphonuclears. In acute nephritis the relative count was not constant; the polymorphonuclears were at first slightly increased, while later the lymphocytes rose in proportion to the total number of cells. The eosinophiles were never increased.

MALIGNANT SCARLET FEVER.

In this classification are considered two cases in which the toxic symptoms completely overshadowed all others, the patients becoming comatose within eighteen hours after the onset; and a third in which there was a persistent high temperature until death, at the end of three weeks. In each of the former cases but one count was obtained, death ensuing on the first and third day respectively. In one of these cases the examination was obtained fourteen hours after the onset, and the count amounted to 6,520. The relative count showed a complete absence of eosinophiles, while the polymorphonuclears were present in the proportion of 68.5, and the lymphocytes of 31.45, per cent. The second patient gave a count of 10,832 leucocytes in the proportion of—

	Per cent.
Polymorphonuclears - - - - -	76.0
Lymphocytes - - - - -	24.7
Eosinophiles - - - - -	0.3

The interesting feature in both of the above cases is the small number of leucocytes present, many writers claiming a leucocytosis of 40,000 in malignant scarlatina. The loss in the eosinophiles follows the rule as seen in scarlatina of moderate intensity, in that their numbers are decreased in proportion to the existing toxemia.

The third example of this type of scarlatina suffered with a concomitant diphtheria, which bars any conclusions, as regards scarlet fever, to be drawn from it. At any rate, it is interesting to note that here, too, the leucocyte count was low, ranging (in four counts taken at intervals during three weeks) from 8,340 to 13,860. Also in the relative count the proportion of eosinophiles was reduced, numbering from 0.16 to 0.24 per cent. of the total white cells.

The polymorphonuclear cells stained poorly and did not show the acidophile tendency as seen in the preceding type. The nuclei showed fragmentation—an indistinct outline—and lay close to the periphery of the cell. The nuclei stained a pale blue, and the meshes of the intranuclear arrangement were loose and in many places transparent. The oxyphile granules in the eosinophiles had a washed appearance, as if these granules had partially dissolved in the cell substance which then took the acid stain.

CASES TREATED WITH ANTISTREPTOCOCCIC SERUM.

Two patients who toward the end of the first week of the disease received 30 c.c. antistreptococcic serum were examined. In each case a count was made immediately before the administration, and then a second count four hours after.

The results obtained were as follows:

	CASE I		CASE II	
	Before Admin- istration	Four Hours After	Before Admin- istration	Four Hours After
Total count	17,160	15,520	20,656	16,410
Relative count —				
Polymorphonuclears ..	82.1%	78.3%	88.4%	80.1%
Lymphocytes	16.8	18.3	8.7	12.3
Eosinophiles	1.1	3.4	3.9	7.6

The changes occurring were thus a diminution in the total number of leucocytes and a relative increase in the eosinophiles and lymphocytes. The increase was particularly noticeable in the eosinophiles, which at the same time appeared smaller than normal. No change was noted in the staining qualities of the

different leucocytes. The diminution in the total number of leucocytes, it should be noted, is similar to that seen after the administration of antitoxin in diphtheria.

GENERAL SUMMARY.

Bowie, considering his *mild* cases under scarlatina simplex, obtained as high a count as 34,900. His average, however, in this class varied from 10,000 to 18,850. As a general rule, he states, the slighter cases have a low leucocytosis. In my own series the changes in the white cell elements, in mild scarlatina, are slight. The white cell count rarely reaches 20,000, and falls back to normal in the third, or even the second, week. A leucocytosis of any intensity is not the rule, nor are there marked changes in the proportions of the different leucocytes. As already suggested, the partial want of harmony between the two series would appear to be due to difference in the grade of case dealt with by Bowie and myself under this heading.

GENERAL SUMMARY.

As regards scarlatina of *moderate* severity, the series here recorded shows a constant leucocytosis of high degree—as a rule from 20,000 to 40,000; it may rise as high as 78,000. In this form the leucocytosis is more marked than that seen in either the mild or fatal cases. The height of the leucocytosis is attained on the third or fourth day after the onset of the disease, from which time it gradually falls to normal at the end of the fifth week. The duration of the leucocytosis varies with the severity of the attack, some cases falling to normal in four weeks, others being protracted into the seventh week.

The early changes are the rapid increase in the polynuclears, rising to as much as 93 per cent. of all the cells. Coincident with the increase of the polynuclears there is a fall in the number of lymphocytes. In uncomplicated cases the polynuclears have attained their maximum in the first week, and then gradually decrease, reaching their normal from the third to the fourth week or later according to the severity of the attack. Early in the first week of the disease the eosinophiles are found below the maximal limits of the normal count, and at times below the minimal

limit. Then there is a rapid increase, which, during the second and third week, may attain a proportion of 19 per cent. of the total leucocytes. The cases showing a good resistance to the disease, and in which the prognosis is good, attain the maximum eosinophile count within the first ten days. The normal is again reached toward the end of the fifth week.

These cases of moderate severity appear to represent in part Bowie's series of cases of scarlatina anginosa. In these anginose cases Bowie found that the polynuclears were increased relatively and absolutely at first, attaining as much as 90 per cent. in one case. His highest counts were obtained on the third and fourth days, and in cases where there were no complications normal was again reached at the end of the third week. The eosinophiles, he noted, were decreased during the first few days. This decrease was evident for a greater length of time in the more severe cases, and an increase was evident only when the tissues were obtaining the upper hand of the toxemia.

In my cases I found that the eosinophiles varied according to the severity, not only in number, but also in appearance. In moderately severe cases, where the tissues were gradually overcoming the attack of the toxins, the oxyphile granules were well marked and crystalline in appearance. If the percentage of the eosinophiles is high, the individual cells are smaller than normal; the nucleus is more compact and stains a deep blue. The polymorphonuclear cells show a strong activity for acid stains. The oxyphile characteristic of these cells varies directly with the intensity of the disease along with the resistance exhibited by the body, or conversely. In severe attacks of scarlatina the prognosis is more favorable in those showing a marked oxyphile nature of the polymorphonuclear leucocytes, along with a high eosinophile count.

This matter of degenerative appearances seen in leucocytes at one or other stage of an infective process deserves attention. Until recently but little mention has been made regarding it in the literature. Arneth's paper in the *Deutsche medicinische Wochenschrift*, Nos. 1 and 2, 1904, is the beginning of a fuller study of the subject. For the present, in the absence of wider

knowledge, it seems to me advisable merely to call attention to the fact that degenerative changes of the nature described are to be met with in the leucocytes of scarlet fever, without attempting to discuss their ultimate nature. The accompanying plate gives, as nearly as I can express it, the varieties in the appearance of the oxyphile granules.

Complications and sequelæ of lymphadenitis, arthritis, varicella, and otitis each show an increase of 4,000 to 12,000 in the number of white cells, the polynuclears showing the greatest change. Nephritis may show either an increase or a decrease in the leucocytosis, depending on the stage and severity of the disease it accompanies. Weakly patients who have had a hard struggle with the original scarlatinal intoxication, and whose resistance has not been brought up to a point to throw off a new attack on the kidneys, show a fall in the number of white cells. On the contrary, patients who during convalescence have a transient, more or less severe, attack of nephritis have an increased leucocytosis.

Reverting again to Bowie, he found that all his cases of nephritis showed an increased leucocytosis, but he notes that the cases of short duration and more favorable prognosis have the highest leucocyte count.

Malignant scarlatina—in which I do not include severe complicated cases, but only those which are intensely affected by the scarlatinal toxin alone—tend to show a low total leucocytosis, and also a diminished eosinophile proportion. Compared with the previous type of the disease, it is thus seen that the leucocytosis varies according to the severity of the disease up to a certain limit, when the extreme toxemia either paralyzes the cell-producing organs, or else causes a cytolytic action. At any rate, there is a diminished number of white cells.

In these malignant cases the polymorphonuclear cells do not show the oxyphile character seen in the cases with a favorable prognosis. The indistinct appearance of the oxyphile granules, with the "washed," pink-stained protoplasm of the few eosinophiles present, would tend to give one the impression that there is cytolysis of this type of cell going on in malignant scarlatina. We no longer find the eosinophiles with the protoplasm densely packed with oxyphile granules, and a deeply stained nucleus.

Bowie lays stress on the low total leucocytosis and the almost total absence of the eosinophiles in unfavorable cases, but he makes no note as to the changes in the staining qualities of the different kinds of cells.

A further study determining the part played by the oxyphile granules is required to clear up the significance of the eosinophilia.

In conclusion, I wish to express my thanks to Dr. Hektoen for valuable references to articles which otherwise would have escaped me, and to Dr. Adami for suggestions and counsel.

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AUTOMATIC THERMO-REGULATOR FOR 20° C. INCUBATORS.*

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At the Laboratory of the Board of Public Works in Harrisburg, Pa., we have recently installed, and are now successfully operating, an automatic thermo-regulator for a 20° C. incubator used in the cultivation of ordinary water bacteria. By its use the incubator can easily be kept at a temperature within limits of 1° C. None of the parts of the regulator are new. The method of regulation is simply the conversion of existing devices to a new purpose.

THE PROCESS.

The method consists of the control of the amount of cooled water which flows by gravity through the outer chamber of the incubator. This is done by placing a thermostat of some pattern in the interior of the incubator. When the air in the incubator becomes too warm, the pointer of the thermostat makes a contact with one of the binding posts, thus forming a circuit between a tandem of ammonium chloride batteries and a clock-work motor. When the circuit is thus made, a magnet in the escapement of the motor releases this and allows the motor to make a half turn, thus opening a balanced valve which controls the supply of cooled water, and at the same time, by means of a shunt, throws the circuit onto the other binding post of the thermostat. When the pointer of the thermostat, as a result of the cooling of the interior of the incubator, makes a contact with the other binding post, the motor is again operated, making another half turn, thus closing the valve. This process continues as the temperature of the interior of the incubator changes. The only things necessary besides the apparatus are that the room temperature shall always be as high as 20° C.; that there shall always be a good supply of ice; and that the motor shall always be wound.

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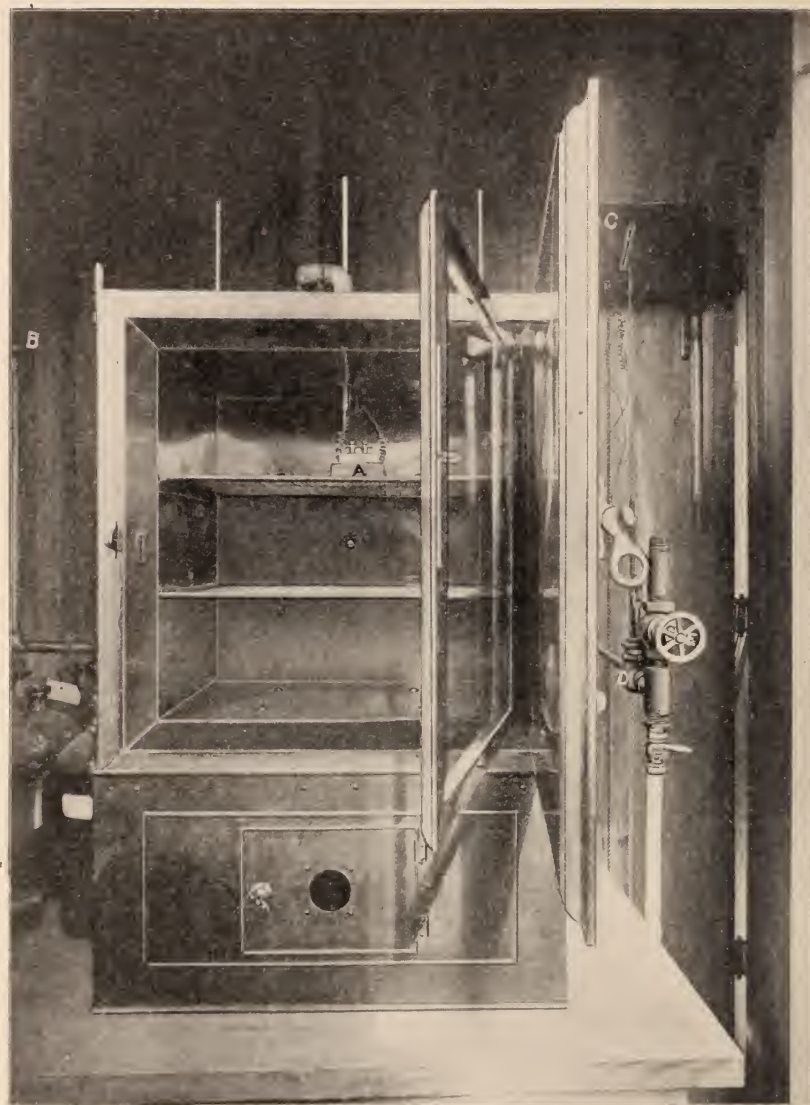


FIG. 1.—20° C. incubator, showing installation of automatic regulator: A, thermostat in place; B, outlet pipe; C, motor; D, inlet valve; E, valve for regulating quantity of water.

ICE WATER.

The cooled water is supplied by passing water from our filtered water tank through a coil of pipes in the ice tank of the refrigerator used for the storage of bacteriological samples, media, etc. The water is piped to the incubator by a three-quarter inch galvanized iron pipe,



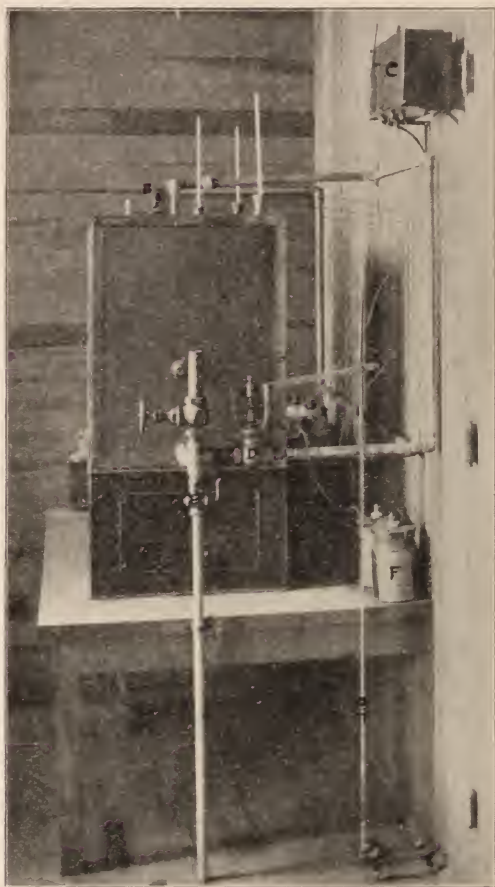
FIG. 2.—Refrigerator, showing piping for purpose of cooling water. Central pipe is only for emptying water from ice tank.



FIG. 3.—Thermostat with expanding bar of hard rubber and brass.

along which occur a valve for regulating the quantity of water and a balanced valve which regulates the time at which the water flows. It is necessary to regulate the quantity of water flowing, in order that too great pressure may not exist on the walls of the incubator. The cold water is introduced into the incubator at the top and led away from the bottom. If the cold water should

be introduced at the bottom, it would remain there, while the warmer water already in the incubator would be forced out at the top, thus causing the bottom of the incubator to be cool while the top was warm. When the cold water enters at the top,



FIGS. 4 and 5.—20° C. incubator, showing thermo-regulator attachments. *B*, outlet pipe (water running in Fig. 5); *C*, mechanical motor; *D*, balance valve; *E*, valve controlling quantity of water; *F*, batteries; *G*, chains between motor and balance valve. (Fig. 4 shows balance valve closed; Fig. 5, open.)

it will naturally pass to the bottom, cooling the warmer water in its passage, thus producing a more even cooling of the interior. There is a by-pass, so that if the water is naturally below 20°

C., it may be passed directly through the incubator without first passing through the refrigerator. The exit pipe from the incubator passes as high as the top of the incubator, and empties into a sewer pipe as an open stream, in order that one can observe the quantity of water flowing, and to relieve the pressure on the walls of the incubator.

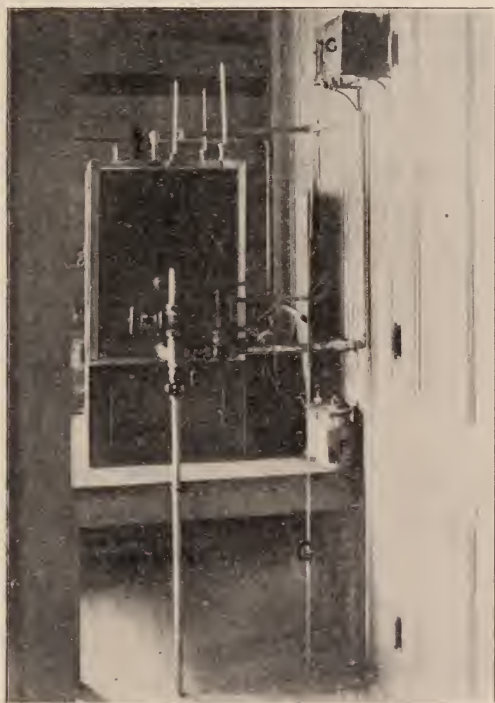


FIG. 5.

THE THERMOSTAT.

The thermostat may be of any of the existing patterns. The one which we use consists of an expanding bar made of hard rubber and brass, firmly bound together and pivoted at one end, the other end being free to move in either direction, making contact with a binding post on either side as the bar expands or contracts. This thermostat is so arranged that both binding posts may be

turned to one side for purposes of adjustment. The expanding bar is connected with the batteries and each of the stationary binding posts to the motor.

THE BATTERIES.

Any battery with sufficient power to move the magnet on the escapement of the motor would suffice. We have a two-cell ammonium chloride battery with carbon cylinder.

THE MOTOR.

The motor is a common clock-work motor, such as is used in the regulation of street steam and furnaces. It is set in motion by the lifting of the magnet attached to the escapement, and when so acted on makes a half turn, thus opening the balanced valve by means of chains passing through pulleys between the balanced valve and motor. At the same time, by means of a shunt, the circuit is thrown onto the other binding post of the thermostat and made ready for the opposite contact. The motor is wound each day by means of a key or crank.

COST.

The cost of the whole apparatus was as follows:

Thermosfat	-	-	-	-	-	-	-	-	\$ 3.00
Motor	-	-	-	-	-	-	-	-	10.00
Valve	-	-	-	-	-	-	-	-	4.00
Batteries, wire, chains, pulleys, etc.	-	-	-	-	-	-	-	-	1.95
									<hr/> \$18.95

Charges for piping, extra.

Our apparatus has been installed about three months, and works admirably within limits of 1° C., and might be made to work more closely. It is so adjusted that when the temperature reaches 20.5° C. the cold water is turned on, and when the temperature falls to 19.5° C. the cold water is shut off. The temperature graduations of the thermostat are not relied upon, but the temperature is observed from accurately graduated thermometers reading between -10° and $+50^{\circ}$ C.

THE INFLUENCE OF ALCOHOL, ETHER, AND CHLOROFORM ON NATURAL IMMUNITY IN ITS RELATION TO LEUCOCYTOSIS AND PHAGOCYTOSIS.*

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THE presence of pathogenic bacteria in perfectly healthy individuals has been repeatedly demonstrated by various observers. The bacillus of tuberculosis, the diplococcus of Fränkel, or the Klebs-Löffler bacillus may be present in the air passages of persons who are not in the least inconvenienced thereby, and do not betray the slightest signs of tuberculosis, pneumonia, or diphtheria. The streptococcus and the various pyogenic staphylococci may be present in the skin or the mucous lining of various orifices of the body without giving rise to any symptoms whatsoever. But at some moment, under certain conditions or in a certain environment, these very organisms, which hitherto had seemed harmless, may suddenly become active, multiply rapidly, and produce a disease that entails serious and even fatal consequences. In this work I have tried to study certain factors that weaken the natural resistance or immunity and allow germs to attack the body.

Before the discovery of the pneumococcus it was thought that cold was the principal cause of fibrinous pneumonia. This led Heidenhain to make some experiments on rabbits. He tried to chill them suddenly by removing them from a hot into a cold atmosphere, but failed to produce pneumonia. Though these animals are not prone to that affection, still, had the specific organism been present, the experiments might have come out differently.

At the present time cold is regarded as only a predisposing cause, or, as Eichhorst calls it, *ein Hilfsmoment für die Infection*. We also know that alcoholism is a predisposing cause, and, if alcohol, why not ether and chloroform, in view of post-operative

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pneumonia and other infections in apparently aseptic cases? It is the influence of alcohol, ether, and chloroform on natural immunity, and especially on the rôle that the leucocytes play in the defense of the organism against various infections that I have studied.

A good deal of work has been done along the same lines, the most recent being that of Snel, who, at the suggestion of Talma, carried out a number of experiments with guinea pigs, relative to the effect of ether, alcohol, and chloral on immunity. He found that those agents suspend immunity temporarily; and, further, that the longer the period of anesthesia, the shorter the process of infection. Snel employed the anthrax bacillus as the infectious material, and in every instance the infection proved fatal to animals, otherwise immune, when they were subjected to narcotics.

Platania—as quoted by Snel—obtained as early as 1889 similar results in frogs, with curare, which he found reduced their resisting power against *Bacillus anthracis*.

Cantacuzène demonstrated the detrimental effects of opium on leucocytes. He observed that guinea-pigs, well immunized against typhoid, when narcotized with opium succumbed readily to typhoid infection. It was observed that the phagocytes did not intervene as promptly as in the cases where opium was not used. Oppel confirmed the results of Cantacuzène by similar experiments. Gheorghiewski, quoted by Metchnikoff, obtained identical results in his experiments with tincture of opium in guinea-pigs. He also found that diapedesis and phagocytosis were impaired; the movements and tactile sensibility were greatly diminished. The animals that were narcotized, though thoroughly immunized, died regularly from the infection, owing, it seemed, to the delayed phagocytosis, in consequence of which the microbes were not destroyed in time.

The ingenious experiments of Besredka show distinctly the rôle which the leucocytes play in defending the organism against poisons. He injected trisulphide of arsenic (which is insoluble and easily recognized as bright orange-colored granules) into the peritoneal cavity of guinea-pigs. This at first caused a dis-

appearance of the leucocytes, followed later by a hyperleucocytosis with predominance of macrophages, which were the principal cells to take up the orange-colored granules of the trisulphide of arsenic. In another experiment he first induced artificially a hyperleucocytosis in the peritoneal cavity, and then injected a lethal dose of the trisulphide, but as in the first experiment, the granules were taken up promptly by the macrophages, and the animal remained unharmed.

In another interesting experiment Besredka demonstrated the capability of the phagocytes to select materials. He injected into the peritoneal cavity trisulphide of arsenic and carmine at the same time, and found that, while a few leucocytes here and there contained orange-colored granules, many of them contained carmine granules. The same phenomenon occurred even when he injected the carmine some time after the arsenic.

My own experiments, begun in 1902, are divided into three separate series, viz., those with alcohol, with ether, and with chloroform. The rabbit was selected as the most suitable animal, The narcotics were usually administered hypodermically, the alcohol and ether in pure form, while the chloroform was shaken up with an equal amount of distilled water, so as to render it less irritating. The average dose of alcohol and ether injected was 1.5–2 gr. to the kilogram of rabbit weight, and of chloroform 1 gr. per kilogram. That dose for alcohol and ether is perfectly safe. In twenty experiments I met with only a single accident, the needle entering a vein, so that ether and some air was injected. The rabbit died instantaneously. But chloroform is not so safe, especially in young animals. In one case two young rabbits succumbed almost immediately after the injection. The rabbits that recovered from the immediate effects of the narcotics all remained well. These experiments were all preliminary. The materials used for inoculation were cultures of pneumococcus and streptococcus.

ALCOHOL SERIES.

EXPERIMENT I.

August 17, 4 P. M.—Rabbit A: white; weight, 2 kg.; leucocytes, 8,600; temperature, 103°. Injected 1 c.c. of a fresh culture of streptococcus hypodermically in ear.

August 18, 4 P. M.—Leucocytes, 15,400; temperature, 101°5.

August 19, 4 P. M.—Leucocytes, 23,300; temperature, 101°4.

The rabbit remained well.

Rabbit B: white and black; weight 2 kg.; temperature, 103°; leucocytes, 8,800. Injected 4 c.c. of alcohol (95 per cent.) and 1 c.c. of streptococcus culture (same as Rabbit A).

August 18, 4 P. M.—Leucocytes, 7,000; temperature, 103°.

August 19, 4 P. M.—Leucocytes, 7,900; temperature, 103°8.

Died in a cachetic state ten days after inoculation.

EXPERIMENT II.

August 28, 3 P. M.—Rabbit A: white; weight, 1 kg.; leucocytes, 14,000. Injected 1 c.c. of streptococcus culture.

August 29, 3 P. M.—Leucocytes, 15,000.

August 30, 3 P. M.—Leucocytes, 9,000.

August 31.—Died sixty hours after inoculation. Smears of heart's blood showed streptococci.

Rabbit B: gray; weight, 1 kg.; leucocytes, 15,000. Received 2 c.c. of alcohol and 1 c.c. of culture. Died in twenty-four hours. Leucocytes one hour ante mortem, 5,500; streptococcus recovered by cultures; smears from blood were negative.

EXPERIMENT III.

August 31, 4 P. M.—Rabbit A: gray; weight, 1¼ kg.; leucocytes, 10,000; temperature, 102°5. Injected 3 c.c. of alcohol and 1/40,000 c.c. of a pneumococcus culture.

September 1, 4 P. M.—Leucocytes, 10,600; temperature, 103°5.

September 2, 4 P. M.—Leucocytes, 19,000; temperature, 103°8.

September 3, 4 P. M.—Leucocytes, 14,000; temperature, 104°.

The number of leucocytes gradually decreased, and the rabbit died at the end of one week. The pneumococcus was found in smears.

Rabbit B: grayish yellow, weight, 1¼ kg.; temperature, 101°5; leucocytes, 8,000. Received 1/40,000 c.c. pneumococcus culture.

September 1, 4 P. M.—Appeared less sick than Rabbit A. Temperature, 104°6; leucocytes, 10,200.

September 2, 4 P. M.—Leucocytes, 18,700; temperature, 105°.

September 3, 4 P. M.—Leucocytes, 22,000; temperature, 105°.

The leucocyte count fell gradually, and the rabbit died at about the same time as Rabbit A.

EXPERIMENT IV.

September 7, 3 P. M.—Rabbit A: gray; weight, 1½ kg., temperature, 102°; leucocytes, 14,000. Received 3 c.c. of alcohol and 0.5 c.c. of pneumococcus culture intraperitoneally. The rabbit died during the night—time unknown. The pneumococcus was found in smears.

Rabbit B: gray; weight 1½ kg.; temperature 102°; leucocytes, 14,600. Received 0.5 c.c. of pneumococcus culture. Died during the night. Diplococcus pneumoniae was found in smears.

EXPERIMENT V.

September 10, 3 P. M.—Rabbit A: black and white; weight, $1\frac{1}{2}$ kg.; temperature, $101^{\circ}5$; leucocytes, 14,000. Injected 3 c.c. of alcohol and 1 c.c. of pneumococcus culture. Died in 22 hours. Leucocytes immediately post mortem, 1,100. An abundance of *Diplococcus pneumoniae* was found in smears.

Rabbit B: black; weight, 2 kg.; temperature, 102° ; leucocytes, 10,500. Received $1\frac{1}{3}$ c.c. of pneumococcus culture.

September 11, 5 P. M.—Temperature, 104° ; leucocytes, 12,400.

September 12, 5 P. M.—Temperature, 104° ; leucocytes, 11,400.

September 13, 4 P. M.—Temperature, $103^{\circ}5$; leucocytes, 18,000.

Rabbit recovered.

EXPERIMENT VI.

September 23, 3 P. M.—Rabbit A: gray: weight $1\frac{3}{4}$ kg.; temperature, 102° ; leucocytes, 8,400. Received $3\frac{1}{2}$ c.c. of alcohol and 1 c.c. of streptococcus (48-hour culture).

September 24, 4 P. M.—Temperature, 105° ; leucocytes, 6,800.

September 25, 4 P. M.—Temperature, $107^{\circ}2$; leucocytes, 2,000.

Died during the night of September 25.

Rabbit B: gray-white; weight, $1\frac{3}{4}$ kg.; temperature, $102^{\circ}5$; leucocytes, 7,700. Received 1 c.c. of streptococcus culture (48-hour).

September 24, 4 P. M.—Temperature, 105° ; leucocytes, 10,300.

September 25, 4 P. M.—Temperature, $104^{\circ}8$; leucocytes, 12,600.

September 26, 4 P. M.—Temperature, 105° ; leucocytes, 21,300.

The rabbit remained well.

EXPERIMENT VII.

October 2, 4 P. M.—Rabbit A: gray; weight, $1\frac{1}{2}$ kg.; temperature 102° ; leucocytes, 10,200. Received 3 c.c. of alcohol and $1\frac{3}{4}$ c.c. of pneumococcus culture.

October 3, 4 P. M.—Temperature, 105° ; leucocytes, 5,300.

October 5.—Died about 1 P. M. Leucocytes ante mortem 7,700. The pneumococcus was found in cultures.

Rabbit B: white; weight, $1\frac{1}{2}$ kg.; temperature, $101^{\circ}8$; leucocytes 9,200. Received $1\frac{3}{4}$ c.c. of pneumococcus culture.

October 3, 4 P. M.—Temperature, $106^{\circ}2$; leucocytes, 11,300.

October 5, 4 P. M.—Temperature, 105° ; leucocytes, 24,000.

October 6.—Died 11 A. M. Leucocytes ante mortem 12,700. At autopsy peritonitis and multiple abscesses of the liver were found. The pneumococcus was found in smears and cultures.

EXPERIMENT VIII.

October 5, 4 P. M.—Rabbit A: gray; weight, $1\frac{1}{2}$ kg.; temperature, 102° ; leucocytes, 11,200. Injected 3 c.c. of alcohol and 3 c.c. of diluted pneumococci blood from Rabbit A, Experiment VII.

October 6.—Died fifteen hours after inoculation. Leucocytes immediately post mortem, 1,800.

Rabbit B: black; weight, $1\frac{1}{2}$ kg.; temperature, $102^{\circ}5$; leucocytes, 9,800. Received the same pneumococcic blood as Rabbit A.

October 6, 4 P. M.—Temperature, $106^{\circ}6$; leucocytes, 22,000.

October 7, 4 P. M.—Died 3 P. M. Leucocytes post mortem (heart's blood), 11,300.

Pneumococci were found in both rabbits.

EXPERIMENT IX.

October 9, 4 P. M.—Rabbit A: black and white; weight, $1\frac{1}{2}$ kg.; temperature, $101^{\circ}5$; leucocytes, 11,800. Received 2 c.c. of alcohol and $1\frac{1}{2}$ c.c. (two loops in salt solution) of pneumococcus culture.

October 10, 4 P. M.—Temperature, 105° ; leucocytes, 2,400.

October 11.—Died thirty hours after inoculation.

Cultures gave positive results.

Rabbit B: white; weight, $1\frac{1}{2}$ kg.; temperature, 102° ; leucocytes, 9,600. Received $2\frac{1}{2}$ c.c. of same mixture of pneumococcus in salt solution as Rabbit A, but this rabbit received 0.5 c.c. more than Rabbit A, owing to error in technique.

October 10, 4 P. M.—Temperature $104^{\circ}5$; leucocytes, 11,100.

October 11.—Died forty-two hours after inoculation.

In spite of the larger dose of culture received, Rabbit B survived his fellow by 12 hours. Pneumococci were found in the cultures.

EXPERIMENT X.

October 14, 4 P. M.—Rabbit A: gray; weight, 2 kg.; temperature, 101° ; leucocytes, 10,900. Received 4 c.c. of alcohol and 2 c.c. of pneumococcus culture.

October 15, 4 P. M.—Temperature $103^{\circ}5$; leucocytes, 24,000.

October 16, 4 P. M.—Temperature 105° ; leucocytes, 5,700. Rabbit appeared quite sick.

October 17, 4 P. M.—Temperature, 105° ; leucocytes, 3,700.

October 18.—Died at 11 A. M.

Smears of blood showed an abundance of pneumococci.

Rabbit B: white; weight $1\frac{3}{4}$ kg.; temperature, 100° ; leucocytes, 12,000. Received 2 c.c. of same culture as Rabbit A.

October 15, 4 P. M.—Temperature, $103^{\circ}5$; leucocytes, 16,000.

October 16, 4 P. M.—Temperature, $103^{\circ}6$; leucocytes, 25,400.

October 17, 4 P. M.—Temperature, 103° ; leucocytes, 27,000.

October 18.—Apparently well.

October 19.—Continued to be well. Recovered.

The following conclusions may be drawn from the foregoing experiments:

That eight out of the ten experiments were positive is ample evidence that alcohol has a detrimental influence upon the resisting power of the animals. The results in Experiments I, IV, V,

and X are striking. Every narcotized animal succumbed to the infection, while all of the control animals completely recovered. In Experiments II, VII, VIII, and IX we see that every control rabbit survived his alcoholized fellow for a certain space of time. And the controls exhibited other signs of resistance; *e. g.*, higher leucocytic counts and better physical signs.

The average leucocytosis of the control animals is markedly greater than that of the narcotized ones. In every instance where the leucocyte count rose considerably above the initial number soon after the infection, and remained high for forty-eight or seventy-two hours, the animal recovered, or at least it remained alive for a variable space of time. But in those animals where the leucocytes did not show a tendency to rise above the initial number, or where they rose for a short time and then fell rapidly, and especially when it fell below the initial count, the animals succumbed.

The relation between the number of leucocytes and the bacteria circulating in the blood will be shown later in the study.

ETHER SERIES.

EXPERIMENT I.

May 25, 4 P. M.—Rabbit A (Belgian hare): weight, 3 kg.; temperature, 101°; leucocytes, 12,000. Received 5 c.c. of ether and 1 c.c. of attenuated pneumococcus culture intratracheally.

May 26, 4 P. M.—Breathing very rapidly; very sick. Temperature, 104°5; leucocytes, 13,500.

May 27.—Died thirty hours after inoculation.

Post-mortem: Both lungs very edematous; left lung grayish in appearance, and floats. Smears of heart's blood show an abundance of pneumococci.

Rabbit B (Belgian hare): weight, 2 kg.; temperature, 101°8; leucocytes; 9,500. Received 1 c.c. of same culture as A intratracheally.

May 26, 4 P. M.—Temperature, 105°5; leucocytes, 16,000.

May 27, 4 P. M.—Temperature, 104°8; leucocytes, 19,300.

May 28, 4 P. M.—Temperature, 105°4; leucocytes, 33,600.

May 29, 4 P. M.—Temperature, 102°5; leucocytes, 18,600.

Recovered.

EXPERIMENT II.

June 5, 3 P. M.—Rabbit A: weight, 2 kg.; temperature, 102°; leucocytes, 11,000. Received 3½ c.c. of ether and 2 c.c. of streptococcus culture of medium virulence.

June 6, 4 P. M.—Temperature, 106°; leucocytes, 16,000.

June 7, 4 P. M.—Temperature, 104°; leucocytes, 6,400.

June 8.—Found dead early in the morning. Cultures made from the blood gave positive results.

Rabbit B (Belgian hare): weight, 2 kg.; temperature, 101°8; leucocytes, 9,800. Received same culture as A (2 c.c.).

June 6, 4 P. M.—Temperature, 104°; leucocytes, 18,000.

June 7, 4 P. M.—Temperature, 104°5; leucocytes, 30,000.

June 8, 4 P. M.—Temperature, 105°2; leucocytes, 32,000.

June 9, 4 P. M.—Temperature, 103°5; leucocytes, 26,000.

The rabbit developed a local abscess, but remained well otherwise.

EXPERIMENT III.

June 11, 4 P. M.—Rabbit A (Belgian hare): weight, 2 kg.; temperature, 102°; leucocytes, 8,500. Received 4 c.c. of ether and 1 c.c. of a streptococcus culture.

June 12, 4 P. M.—Temperature, 102°; leucocytes, 8,000.

June 13, 4 P. M.—Temperature, 103°; leucocytes, 16,000.

June 15, 4 P. M.—Temperature, 102°5; leucocytes, 8,200.

June 16, 4 P. M.—Temperature, 103°; leucocytes, 7,100.

June 17, 4 P. M.—Temperature, 103°; leucocytes, 7,200.

June 18.—Died early in the morning; streptococcus found abundantly in blood.

Rabbit B (Belgian hare): weight, 2¼ kg.; temperature, 103°; leucocytes, 8,300. Received 1 c.c. of same culture as A.

June 12, 4 P. M.—Temperature, 103°5; leucocytes, 9,000.

June 13, 4 P. M.—Temperature, 103°5; leucocytes, 11,000.

June 15, 4 P. M.—Temperature, 104°5; leucocytes, 14,000.

June 16, 4 P. M.—Temperature, 104°; leucocytes, 14,600.

June 17, 4 P. M.—Temperature, 103°5; leucocytes, 11,000.

Rabbit remained well.

EXPERIMENT IV.

June 25, 4 P. M.—Rabbit A: weight, 1½ kg.; temperature, 103°; leucocytes, 10,500. Received 3 c.c. of ether and 1 c.c. of pneumococcus culture intratracheally.

June 26, 3 P. M.—Temperature, 101°6; leucocytes, 9,300.

June 27, 4 P. M.—Temperature, 102°; leucocytes, 7,500.

June 28.—Found dead in morning. Autopsy: lung edematous; infarct in left lower lobe; areas of acute congestion; pneumococcus present in smears.

Rabbit B: weight, 1¼ kg.; temperature, 102°5; leucocytes, 14,600. Received 1 c.c. of same culture as A, intratracheally.

June 26, 3 P. M.—Temperature, 105°2; leucocytes, 19,600.

June 27, 4 P. M.—Temperature, 103°5; leucocytes, 14,000.

June 29, 4 P. M.—Temperature, 103°; leucocytes, 17,000.

Died July 8. Examination for pneumococci in blood gave negative results. Lung edematous; no congestion.

EXPERIMENT V.

July 16, 3 P. M.—Rabbit A: weight, 2 kg.; temperature, 102°6; leucocytes, 9,800. Injected 4 c.c. of ether and 3 c.c. of pneumococcus culture intravenously. Died eight hours after inoculation. Pneumococci present in blood (smears).

Rabbit B: weight, 2 kg.; temperature, 102°; leucocytes, 11,800. Received 3 c.c. of same culture intravenously. Died fifteen hours later. Bacteriologic examination gave positive results.

EXPERIMENT VI.

July 20, 3 P. M.—Rabbit A: weight, 2½ kg.; temperature, 102°4; leucocytes, 10,500. Received 5 c.c. of ether and culture of pneumococcus intratracheally.

July 21, 3 P. M.—Temperature, 104°6; leucocytes, 8,000.

July 22.—Died early in the morning. Smears from heart's blood showed pneumococcus with well-defined capsules.

Rabbit B: weight, 3 kg.; temperature 103°; leucocytes, 11,000. Received in the trachea same culture (No. 100) as Rabbit A.

July 21, 3 P. M.—Temperature, 104°; leucocytes, 18,000.

July 22, 3 P. M.—Temperature, 106°5; leucocytes, 34,000.

July 23, 3 P. M.—Temperature, 104°6; leucocytes, 22,000.

The rabbit recovered.

EXPERIMENT VII.

July 22, 4 P. M.—Rabbit A: weight, 3¼ kg.; temperature, 102°5; leucocytes, 12,000. Received 6 c.c. of ether and 1 c.c. of pneumococcus culture hypodermically.

July 23, 4 P. M.—Temperature, 106°5; leucocytes, 14,100.

July 24, 4 P. M.—Temperature, 107°; leucocytes, 15,000.

July 25, 4 P. M.—Temperature, 105°5; leucocytes, 15,600.

Rabbit died August 1. No autopsy.

Rabbit B: weight, 2¼ kg.; temperature, 102°; leucocytes, 9,800. Received hypodermically 1 c.c. of same culture as A.

July 23, 4 P. M.—Temperature, 103°8; leucocytes, 10,000.

July 24, 4 P. M.—Temperature, 102°; leucocytes, 11,000.

July 25.—Died in the morning. Pneumococci present in smears.

EXPERIMENT VIII.

July 27, 4 P. M.—Rabbit A: weight, 2 kg.; temperature, 101°6; leucocytes, 11,500. Received 4 c.c. of ether and an intratracheal injection of 1 c.c. of pneumococcus culture.

July 28, 4 P. M.—Temperature, 106°2; leucocytes, 5,000.

July 29.—Died during the day. Blood examination gave positive results. Lungs were edematous.

Rabbit B: weight, 2¼ kg.; temperature, 102°5; leucocytes, 9,200. Ether inhalation for fifteen minutes; semiconsciousness. Received 1 c.c. of pneumococcus culture in the trachea.

July 28, 4 P. M.—Temperature, 104°5; leucocytes, 11,500.

July 29, 4 P. M.—Temperature, 104°5; leucocytes, 9,000.

Died August 2. Autopsy: Lungs edematous; serofibrinous peritonitis; pneumococcus present in exudate (cultures).

Rabbit C, control: weight, 2½ kg.; temperature, 101°5; leucocytes, 8,900. Received 1 c.c. of pneumococcus culture (same as A and B) in the trachea.

July 28, 4 P. M.—Temperature, 106°; leucocytes, 16,000.

July 29, 4 P. M.—Temperature, 105°5; leucocytes, 6,000.

July 30, 4 P. M.—Temperature, 102°; leucocytes, 7,200.

Died August 1. Pneumococcus present in smears.

EXPERIMENT IX.

July 30, 4 P. M.—Rabbit A: weight, 1 kg.; temperature, 102°; leucocytes, 13,000. Received 2 c.c. of ether and 0.5 c.c. of pneumococcus culture intratracheally. Died twelve hours later. Pneumococcus present in the blood.

Rabbit B: weight, 1 kg.; temperature, 101°8; leucocytes, 14,200. Ether by inhalation for 15 minutes and 0.5 c.c. of same culture as A intravenously. Died eighteen hours later. Pneumococci present in the blood.

Rabbit C: weight, 1 kg.; temperature, 102°5; leucocytes, 12,000. Received 0.5 c.c. of pneumococcus culture (same as A and B) intratracheally. Died about the same time as B. Pneumococci present.

EXPERIMENT X.

August 6, 9 A. M.—Rabbit A: weight, 1¾ kg.; temperature, 101°4; leucocytes, 12,000. Received 3.5 c.c. of ether and 1 c.c. pneumococcus culture intratracheally.

August 7, 9 A. M.—Temperature, 105°; leucocytes, 10,600.

August 8, 9 A. M.—Temperature, 105°5; leucocytes, 6,400.

Died August 9. Lung edematous and congested in areas. Pneumococci present in smears.

Rabbit B: weight, 1½ kg.; temperature, 102°; leucocytes, 9,500. Received 1 c.c. of pneumococci (same culture as A) in the trachea.

August 7, 9 A. M.—Temperature, 105°; leucocytes, 9,000.

August 8, 9 A. M.—Temperature, 106°; leucocytes, 18,200.

August 10, 4 P. M.—Temperature, 105°4; leucocytes, 40,000.

August 11, 4 P. M.—Temperature, 103°; leucocytes, 26,000.

The rabbit made a good recovery.

The foregoing experiments show that ether, administered in doses similar to those of alcohol, is still more positive in its effect. The difference in leucocytosis between the etherized rabbits and the controls is also more marked in the last than in the alcohol series.

In Experiment III, Rabbit B recovered with a maximum leucocytosis of 14,000 and an initial count of 8,300, while the narcotized rabbit (A), with a similar initial count and a leucocytosis

on the second day of 16,000 (which, however, fell rapidly), died. One might presume from this result that, if Rabbit A, with a leucocytosis of 16,000 and infected with the same dose of culture as Rabbit B with a leucocytosis of only 14,000, died, while the latter completely recovered, the narcotic has not only a detrimental effect upon the leucocytes numerically, but that it also affects their function.

In Experiment VII, Rabbit A, narcotized, weighed $3\frac{1}{4}$ kg.—a very powerful animal. Although it received the usual dose of ether, it was not completely narcotized. The control weighed only $2\frac{1}{4}$ kg.—a difference of 1 kg.—but they received an equal dose of culture. This test was undertaken purposely, because, had the experiment turned out differently, its value would have been increased.

CHLOROFORM SERIES.

EXPERIMENT I.

October 21.—Rabbit A: weight, 2 kg.; temperature, 102° ; leucocytes, 7,000. Received 2.5 c.c. of chloroform and 1.5 c.c. of a 48-hour streptococcus culture hypodermically in ear. Died the following night. Streptococcus found in blood.

Rabbit B: weight, 2 kg.; temperature, $102^{\circ}5$; leucocytes, 8,200. Received 1.5 c.c. of same culture as A.

October 22, 4 P. M.—Temperature, 105° ; leucocytes, 13,200.

October 23, 4 P. M.—Temperature, 106° ; leucocytes, 12,900.

October 24, 4 P. M.—Temperature, $105^{\circ}5$; leucocytes, 23,000.

Rabbit recovered.

EXPERIMENT II.

October 26, 4 P. M.—Rabbit A: weight, $1\frac{3}{4}$ kg.; temperature, $102^{\circ}5$; leucocytes, 14,200. Kept under chloroform for thirty minutes. Received 2.5 c.c. of streptococcus culture subcutaneously in ear.

October 27, 4 P. M.—Temperature, $105^{\circ}5$; leucocytes, 7,400.

October 28.—Died in the morning. Autopsy: Bloody exudate in pleural and peritoneal cavities; streptococci present in blood.

Rabbit B: weight, $1\frac{1}{2}$ kg.; temperature, 102° ; leucocytes, 8,400. Received 2 c.c. of same culture subcutaneously in ear.

October 27, 4 P. M.—Temperature, $104^{\circ}8$; leucocytes, 8,200.

October 28, 4 P. M.—Temperature, $103^{\circ}5$; leucocytes, 23,400.

October 29, 4 P. M.—Temperature, 103° ; leucocytes, 23,000.

Remained well.

EXPERIMENT III.

November 2, 4 P. M.—Rabbit A: weight, $2\frac{1}{4}$ kg.; temperature, 100° ; leucocytes, 9,400. Received 2.5 c.c. of chloroform and 1 c.c. of streptococcus culture hypodermically in ear.

- November 3, 4 P. M.—Temperature, 102°: leucocytes, 5,800.
- November 4.—Died in the forenoon. Cultures from heart's blood positive.
- Rabbit B: weight, 2½ kg.: temperature, 101°: leucocytes, 10,100. Received 1 c.c. of same culture as A hypodermically in ear.
- November 3, 4 P. M.—Temperature, 104°: leucocytes, 22,800.
- November 4, 4 P. M.—Temperature, 105°: 20,000.
- November 5, 4 P. M.—Temperature, 102°: leucocytes, 21,000.
- Rabbit recovered.

EXPERIMENT IV.

- November 11, 4 P. M.—Rabbit A: weight, 2 kg.: temperature, 101°5: leucocytes, 11,400. Kept under chloroform for thirty minutes, but was not completely under. Received 2 c.c. of streptococcus culture.
- November 12, 4 P. M.—Temperature, 104°: leucocytes, 24,200.
- November 13, 4 P. M.—Temperature, 105°: leucocytes, 22,600.
- November 14, 4 P. M.—Temperature, 105°: leucocytes, 16,500.
- Had a marked erysipelas of ear. Leucocytes fell gradually, and animal died November 20. Streptococcus in blood.
- Rabbit B: weight 2 kg.: temperature, 101°5: leucocytes, 13,100. Received 2 c.c. of same culture.
- November 12, 4 P. M.—Temperature, 101°5: leucocytes, 18,100.
- November 13, 4 P. M.—Temperature, 103°: leucocytes, 21,200.
- November 14, 4 P. M.—Temperature, 103°: leucocytes, 20,900.
- The leucocytes fell gradually, and the rabbit died November 22. Streptococcus found in blood.

EXPERIMENT V.

- November 19, 4 P. M.—Rabbit A: weight 2 kg.: temperature, 102°: leucocytes, 18,200. Kept under chloroform thirty minutes. Received 2 c.c. of pneumococcus culture of low virulence.
- November 20, 4 P. M.—Temperature, 105°: leucocytes, 20,200.
- November 21, 4 P. M.—Temperature, 105°: leucocytes, 23,400.
- November 23, 4 P. M.—Temperature, 104°: leucocytes, 15,800.
- November 24, 4 P. M.—Temperature, 105°: leucocytes, 17,800.
- November 25, 4 P. M.—Temperature, 102°5: leucocytes, 18,000.
- November 27, 4 P. M.—Temperature, 102°: leucocytes, 18,000.
- November 28, 4 P. M.—Temperature, 102°5: leucocytes, 25,000.
- Recovered.
- Rabbit B: weight, 2 kg.: temperature, 102°5: leucocytes, 14,300. Received same culture and dose as A.
- November 20, 4 P. M.—Temperature, 104°2: leucocytes, 19,200.
- November 21, 4 P. M.—Temperature, 104°: leucocytes, 24,200.
- November 23, 4 P. M.—Temperature, 103°5: leucocytes, 13,400.
- November 24, 4 P. M.—Temperature, 104°: leucocytes, 13,600.
- November 25, 4 P. M.—Temperature, 103°: leucocytes, 13,000.
- November 27, 4 P. M.—Temperature, 102°5: leucocytes, 19,000.
- November 28, 4 P. M.—Temperature, 103°: leucocytes, 16,000.
- Rabbit recovered.

The results of the chloroform series are not unlike those obtained with alcohol and ether. The last experiment might have come out differently had Rabbit A been more fully anesthetized. Owing to certain accidents in the course of the chloroform series, the narcotic was employed very cautiously, and the rabbit was allowed to come out of its influence several times during the half-hour. It seems that to produce a decided effect it is necessary to employ enough of the narcotic chosen, to produce complete narcosis, even if that should last only a short time. And I have reason to believe that the longer the narcotization lasts, the more complete and the longer is the suspension of immunity.

It may be well to state here that in the foregoing experiments only comparative results were sought, and the complete recovery of all the control animals was not at all anticipated, since the infectious materials used in many instances were surely many times the lethal dose.

These experiments appear to show that the narcotics in question have a decidedly unfavorable effect on the resistance of rabbits to infections. It does not make any difference how the narcotic is administered. As long as the necessary quantity is absorbed by the system the results are the same. This might well serve to explain post-operative pneumonia, and the drunkard's predisposition to pneumonia, as also the especially severe character of the disease in such persons. The susceptibility of alcoholics to tuberculosis is also an established clinical fact.

Impressed with the idea that the number and quality of the leucocytes have a certain connection with immunity, it seemed desirable to make a series of careful leucocyte counts of drunkards' blood. The following observations have been made on inmates of the Washingtonian home, an institution conducted especially for the care of those addicted to the use of alcohol and other narcotics. I am greatly indebted to the management of that worthy institution for the privilege and especially to Drs. Smith and Goodsmith, of the house staff, for many courtesies.

TABLE I.
LEUCOCYTE COUNTS OF SIXTY ALCOHOLICS.

No.	Name	Age	Occupation	Steady Drinker No. Years	Periodi- cal Drinker No. Years	Leucocyte Count
1	A. W. G.	43	Merchant	20		4,600
2	W. G. C.	40	Salesman		20	8,200
3	O. M.	44	R. R. employee		16	6,200
4	G. P. T.	43	Bartender	15		5,600
5	D. M.	52	Trav. salesman		25	4,200
6	T. R.	44	City employee	15		5,600
7	F. E. L.	35	Newspaper man	10		4,000
8	C. O. J.	37	Engineer	17		5,300
9	T. M.	34	Waiter	15		5,600
10	A. W.	44	Harness-maker		20	3,550
11	J. E. Q.	44	Waiter	25		2,100
12	P. M. I.	30	Letter-carrier	12		4,300
13	J. H. B.	30	Bookkeeper	12		5,400
14	G. E. C.	39	Board of Trade	20		4,000
15	E. M.	42	Lumberman	20		4,700
16	Erig. M.	32	Telegrapher		10	9,700
17	G. D.	52	Laborer	30		3,400
18	J. E. J.	32	Laborer	15		4,000
19	C. T. K.	46	Jeweler		18	9,750
20	G. F. G.	39	Grocer	12		5,400
21	G. E. C.	45	Newspaper man	25		4,600
22	S. P.	34	Laborer	10		5,050
23	M. C. M.	41	Tailor		20	6,200
24	P. T. M.	46	Solicitor	30		4,300
25	M. J. M.	24	Butcher		6	5,050
26	A. M.	53	Saloonkeeper	20		3,700
27	C. O. E.	31	Painter	12		6,500
28	A. M. L.	49	Bookkeeper	20		5,200
29	P. McE.	47	City employee	20		3,600
30	J. R.	37	Laborer	20		6,500
31	G. W. S.	40	Salesman	20		4,700
32	J. P. P.	61	Salesman		40	6,900
33	L. P. C.	37	Farmer	15		4,800
34	J. A. A.	31	Painter	12		4,700
35	A. W.	42	Barber	22		5,000
36	W. E. P.	42	R. R. employee	20		5,500
37	R. J. F.	49	Janitor		15	6,800
38	F. B.	41	Metal-worker	15		4,500
39	J. M.	36	Teamster		18	4,300
40	G. W. M.	45	Painter	18		3,800
41	F. T.	35	Actor	15		6,300
42	J. B.	50	Grocer	25		6,750
43	J. G. C. C.	31	Painter	12		5,800
44	F. U.	36	Bookbinder	10		4,850
45	J. D. C.	48	Trav. salesman	30		5,100
46	J. M.	32	Laborer	10		7,400
47	C. D.	44	Solicitor		20	6,300
48	J. E. M.	41	Woodworker	15		5,400
49	A. J.	30	Peddler	10		6,800
50	H. E. M.	42	Printer	22		4,600
51	F. E.	48	Clerk		10	8,000

TABLE I.—*Continued.*

No.	Name	Age	Occupation	Steady Drinker No. Years	Periodi- cal Drinker No. Years	Leucocyte Count
52	J. T. C.	58	Painter		30	7,100
53	H. A.	62	Merchant		35	5,700
54	A. H.	40	Barber	20		8,000
55	W. W. H.	34	Coachman	10		6,000
56	T. R.	41	Teamster	18		5,300
57	R. T.	38	Grocer	15		6,200
58	J. S.	36	Tailor	12		5,000
59	C. A.	55	Merchant	25		4,900
60	P. O.	42	Plumber		20	5,600
Average, 5,300						

The average number of leucocytes for the individual is 5,300. The average normal number of leucocytes per cubic millimeter is, according to the best modern authorities, 7,500, which means a diminution of 2,200 in the average alcoholic. Of the individuals 92 per cent. fall below 7,500; 72 per cent. below 6,000; 44 per cent. below 5,000; 12 per cent. below 4,000, and 2 per cent. below 3,000; 8 per cent. have above the normal number.

Differential counts made in twenty-five individuals show no change in the ratio of the different forms of leucocytes:

TABLE II.
DIFFERENTIAL LEUCOCYTE COUNT.

No.	Polynu- clear	Lympho- cytes	Large Mononu- clears	Eosino- philes	No.	Polynu- clear	Lympho- cytes	Large Mononu- clears	Eosino- philes
1	75.0%	15.0%	10.0%	0.5%	15	66.0%	29.5%	4.0%	0.5%
2	57.0	30.0	11.5	1.5	16	74.0	20.0	6.0	...
3	72.0	23.0	4.0	1.0	17	82.0	12.0	6.0	...
4	80.0	15.0	3.0	2.0	18	66.0	25.0	8.5	0.5
5	63.5	24.0	10.0	2.5	19	72.0	16.5	9.5	1.5
6	75.0	17.0	8.0	...	20	67.5	23.5	7.5	1.5
7	84.0	11.0	5.0	...	21	77.5	15.0	6.0	1.5
8	77.0	15.5	6.5	1.0	22	71.0	19.0	10.0	...
9	63.5	26.5	8.0	2.0	23	76.0	18.5	4.5	1.0
10	72.0	22.0	6.0	...	24	79.0	13.5	6.0	1.5
11	72.0	15.0	8.0	5.0	25	69.0	19.0	11.5	0.5
12	67.0	22.5	7.5	3.0	Av'ge	72.0	19.0	7.0	1.02
13	72.5	21.0	6.5	...					
14	73.0	20.0	6.5	0.5					

All the blood specimens were taken at about 11 A. M.—that is, four hours after the regular breakfast hour, 7 A. M. To prove whether four hours was a sufficient interval, I made a number of counts at 6 and at 6:30 A. M. in the same individuals that I examined at 11 A. M., and found no marked difference in the number of leucocytes. According to von Limbeck, the number of leucocytes in healthy males at 11 A. M.—the same hour as my counts were made—is 7,660, making the difference between the normal and the number found by me still greater. It is interesting to note that there is an appreciable difference in the average leucocyte counts of the periodical and the steady drinker. The latter has 5,100, while the periodical drinker has 6,500—a difference of 1,400 leucocytes.

One might attempt to explain the foregoing observations by assuming that alcohol has an unfavorable effect upon the leucocytogenic centers (*i. e.*, bone marrow and lymphatic glands), so that in the habitual inebriate, in whom the leucocyte-producing tissues are constantly under the influence of the toxic agent, there is a smaller output of white blood corpuscles, while in the periodical drunkard the same tissues have a chance to recuperate between the periods of intoxication, and are therefore better able to produce leucocytes.

THE NUMERICAL RELATIONS OF CIRCULATING BACTERIA TO LEUCOCYTES.

To ascertain the numerical relationship between the circulating bacteria (pneumococci) and the leucocytes, cultures and leucocyte counts were made at the same time. These observations were carried on in conjunction with Dr. Rosenow, who made the counts of the colonies.

OBSERVATION I.

A rabbit etherized and infected twenty-four hours previously with pneumococcus has a temperature of 103°5 F.; leucocytes, 24,000. Blood cultures made at the same time gave 168 colonies to the loop of blood, that being the average of twelve loops. Forty-eight hours later, before the rabbit died, the leucocyte count was 3,700, and the colonies per loop numbered 46,500 from an average of twelve loops.

OBSERVATION II.

A rabbit etherized and infected forty-eight hours previously with pneumococcus has a temperature of 105° , a leucocytosis of 17,000, and blood cultures made at the same time gave 139 colonies per loop from an average of twelve loops. Twenty-four hours later, immediately before the death of the animal, there was a leucocyte count of 1,160 and 142,900 colonies to the loop of blood from cultures made at the same time.

OBSERVATION III.

A rabbit sick from pneumococcus infection, with a leucocyte count of 27,000, gave only 38 colonies to the loop of blood from an average of twelve loops.

From these observations we may conclude that the presence of leucocytes in the circulation in large numbers limits the numbers of the circulating bacteria, but that as soon as the leucocytes diminish in number there is a rapid rise in the number of bacteria.

In the experiments with alcohol, ether, and chloroform, there were some rabbits that were narcotized, and that had about the same number of leucocytes as the control rabbits, shortly after a certain infection; but those treated with the narcotics either succumbed or did not resist the infection as well as the controls. Now, the question arises whether it is simply the hyperleucocytosis that protects the animal against certain poisons, or whether it also depends upon the quality (chemiotactic and phagocytic) of the individual corpuscles. From the several experiments given above, it would seem that the protection of the animal against various infections depends upon both the number and quality of the white blood corpuscles, otherwise, when both the narcotized and the control rabbit had the same leucocyte count, being infected with the same amount of culture and kept under the same conditions, why should only the former die? To test this possibility, I have undertaken to find out whether the narcotics exert any influence upon the phagocytic action of leucocytes.

In determining the phagocytic qualities of the cells, it was necessary to select some substance which they would take up and which could be easily recognized within the cell. I first thought

of the trisulphide of arsenic, which was especially prepared by Besredka for his experiments described above. But this was abandoned because of the toxic properties of the drug. I then tried powdered charcoal, insoluble Prussian blue and carmine. The latter was finally selected, though it seemed to me that the charcoal and the blue were taken up more readily by the phagocytes. However, the color of those substances made it difficult to separate them from the cell granules and nuclei, especially when treated with acetic acid.

EXPERIMENT I.

Rabbit A received intraperitoneally 3 c.c. of an emulsion of carmine in distilled water by means of a Pasteur pipette. Twenty-four hours later some peritoneal exudate was withdrawn and examined fresh and in acetic acid. Carmine granules were found in about 20 per cent. of the leucocytes. Many of the phagocytes contained as much as ten granules of carmine. In certain fields of the microscope there were as many as 30 per cent. of the leucocytes containing red granules.

Rabbit B was etherized hypodermically and injected intraperitoneally with the same emulsion of carmine and the same amount as in Rabbit A. Twenty-four hours later the exudate from the peritoneum showed that only 5-6 per cent. of the leucocytes contained carmine granules.

EXPERIMENT II.

Rabbit A was injected intraperitoneally with carmine in the same manner as in the previous experiment. The peritoneal exudate was withdrawn twenty-four hours later, and on examination 14 per cent. of the leucocytes were found to contain granules of carmine. Some of them contained as many as eight and ten distinct granules.

Rabbit B was narcotized with alcohol hypodermically and received the same amount of carmine in the peritoneal cavity as Rabbit A. Twenty-four hours later some of the peritoneal exudate was withdrawn and examined as in the previous experiments. It was found that only six out of a hundred leucocytes contained carmine granules. In this experiment the rabbit received a sec-

and smaller dose of alcohol four hours after the first one, owing to the quick recovery of the animal from the effects of the first dose.

CHEMIOTACTIC INFLUENCE OF ALCOHOL ON LEUCOCYTES.

A capillary tube filled with alcohol was inserted subcutaneously in the ear of a rabbit, and another capillary tube filled with bouillon was inserted in the other ear. Twenty-four hours later the tubes were removed and examined under the microscope. The bouillon capillary was filled with leucocytes from the inserted end up to a considerable distance, while the alcohol capillary was absolutely free from leucocytes.

In a second experiment two capillary tubes—one containing alcohol and the other bouillon—were inserted subcutaneously in one ear of a rabbit, only 2 cm. apart. When removed twenty-four hours later and examined under the microscope, the same phenomenon as in the first experiment was observed—*i. e.*, the bouillon tube was filled at the inserted end with leucocytes, while the one containing the alcohol was absolutely free from leucocytes.

From the latter experiments we may conclude that there is a negative chemiotaxis between the leucocytes and alcohol, and this may help to explain why the leucocytes do not appear in as great numbers in infected animals that have also been alcoholized as in those that were not; and those leucocytes that do circulate in the blood stream which contains alcohol were not able to perform their phagocytic function so well, as shown in some of the above experiments. This will perhaps also explain the difference in the leucocyte counts found in the periodical and steady drinkers.

It might be reasonable to suppose that ether and chloroform would give similar results. I have omitted to test the latter narcotics, because they are so easily evaporated, and the results could not have been considered conclusive.

In summing up the results of this work, the following conclusions may be drawn:

1. That alcohol, ether, and chloroform have a decidedly detrimental influence on the natural defenses against infection, and that this lowering or suspension of the resisting power of the

animal is not due to any apparent organic lesions, since no changes in any of the vital organs were discoverable in any of the animals, except such as were caused by the infectious material employed.

2. The narcotics appear to affect directly the substance or substances which inhibit the growth and toxic action of bacteria in the normal animal, and these substances are either the leucocytes themselves or something derived from them or both. Snel has shown in his work that alcohol and ether suspend also conferred immunity against a specific microbe.

3. The period of detrimental action of the narcotic depends largely on the amount administered, the depth of narcosis, and the rapidity of its elimination from the system. Some of the narcotized animals that were employed in other experiments with infectious material twenty-four hours later (*i. e.*, after narcotization) fared as well as the new rabbits.

There are several other questions to be settled in connection with this theme, but they will be left for a separate study.

In closing, I wish to extend my sincere thanks to Professor Hektoen for numerous valuable suggestions and courtesies. I wish to thank also Dr. Rosenow for many kindnesses and favors.

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AGGLUTINATION OF TYPHOID AND PARATYPHOID BACILLI BY VARIOUS IMMUNE SERA.*

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SUMMARY.

INTRODUCTION.

PERHAPS no question in recent years has aroused more interest from a practical standpoint than the diagnosis of disease by the agglutination reaction. When first applied to the diagnosis of typhoid fever, and for some time subsequently, the reliability of this procedure was unquestioned. During the general application, however, contradictory results were occasionally met with

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which made it necessary to modify the interpretation of the occurrence or non-occurrence of the reaction. Since the recognition of paratyphoid fever as a distinct clinical entity caused by an organism resembling the typhoid bacillus, both culturally and in its agglutination reactions, the question of the value of such tests as a means of diagnosis of these diseases became still more complicated.

These experiments were suggested by Dr. Hektoen in January, 1903, and at that time no satisfactory conclusions could be drawn from the diverse results reported by different investigators. A few reports throwing light upon this subject have subsequently appeared, and reference to these will be made in various parts of this paper.

My experiments concern the effect of immune human and animal sera on several strains of typhoid, paratyphoid, and colon bacilli.

TECHNIC.

The human blood was obtained by aspiration with an aseptic 10 c.c. Luer syringe from the median vein of the arm. The area was cleansed with ether followed by a 1:1,000 solution of bichloride of mercury, and 95 per cent. carbolic acid was then applied with a cotton swab over the site of puncture and allowed to remain fifteen seconds before removal with alcohol. After constricting the arm close to the shoulder, the needle was inserted into the distended vein against the current, and the blood withdrawn. The latter was placed in a sterile test-tube and allowed to stand for six hours at room temperature, after which time the serum was poured off into another sterile test-tube and kept for use.

The tedious process of obtaining rabbit's blood from a vein in the ear was early replaced by direct aspiration from the heart. The animal was carefully anesthetized with chloroform and with the usual aseptic precautions, the needle being inserted directly backward in the second right interspace close to the margin of the sternum. Ten c.c. of blood can be quickly withdrawn without deleterious effects.

The diagnosis of typhoid fever, in nearly all cases where the serum was used for experimental purposes, was confirmed by the isolation of the typhoid bacillus from the blood at the same time that the serum was collected.

The macroscopic method of agglutination was used, inasmuch as it is more practical and less laborious than the microscopic method, and bids fair to supplant the latter in clinical work as soon as its application becomes generally known. An emulsion of bacilli was prepared by washing a twenty-four hour growth from the surface of an agar slant culture with a 1 per cent. solution of NaCl. The growth of an agar-slant culture of typhoid bacilli in 7 c.c. of solution was used as a standard of turbidity; emulsions obtained from

the growth of more vigorous organisms were correspondingly diluted. One c.c. of an emulsion thus prepared was mixed in a 4 c.c. tube with an equal quantity of serum diluted with distilled water. Positive results were recorded when a visible formation of flocculi occurred in the mixture after standing for two hours at room temperature. In the subsequent tables the numbers represent the greatest dilutions of the sera with which positive reactions were obtained. For obvious reasons, reactions occurring in dilutions less than 1:40 are not considered. Emulsions of bacilli killed by heat or various antiseptics, formalin, thymol, etc., are nearly as effective as emulsions of living bacilli, and are obviously far more safe to handle; but as the earlier experiments were performed with living bacilli, this method was adhered to in order that the results might be uniform.

B. typh. Nos. I, IV, VI, VII, XII, XV, XXX, and 238 were typical typhoid bacilli isolated from the blood of cases of typhoid fever. B. typh. No. V and B. paratyph. No. X, Eustis, Samuels, and Buxton, were obtained for me by Dr. Hektoen. B. typh. No. III and B. coli Nos. IV and X were furnished by Dr. Gehrman, who isolated them from the urine of typhoid patients. B. paratyph. Smith and Scott were obtained from Dr. H. G. Wells. Grateful acknowledgment is hereby made for these favors.

CLASSIFICATION OF BACILLI.

For convenience of reference, the following classification has been arranged, based principally on the work of Schottmüller, Durham, Cushing and Buxton.

BACILLI MORE OR LESS MOTILE—NEGATIVE TO GRAM'S STAIN.

(Gelatin not liquefied. Differences of growth upon agar, gelatin, and bouillon principally of degree. Potato unreliable. Indol production variable.)

B. coli group.—Ferment lactose with production of acidity in lactose bouillon. Glucose bouillon fermented in 24 hours with visible gas production. Milk coagulated. Neutral red agar—permanent yellow color in 24 hours. Indol production fairly constant.

B. cholerae suis, enteritidis, or intermediate group.—Lactose not fermented. Glucose bouillon fermented in 48–72 hours with visible gas production. Indol production not constant. Litmus milk initial acidity in 24–48 hours.

a) *Paratyphoid group A*. Remains acid; average 1 per cent. on tenth day; no opalescence. Neutral red agar—yellow color in 3 days; turns red in 5–7 days. Glucose bouillon fermented in 72 hours.

b) *Paratyphoid group B*. Becomes alkaline in 5 days; increases with slight opalescence in 10 days. Neutral red agar—permanent yellow in 48 hours. Glucose bouillon fermented in 48 hours. Infections clinically typhoidal.

B. typhoid group.—Lactose not fermented. Glucose fermented without visible gas production. Neutral red agar—unchanged. Litmus milk:

a) Usual type: acidity permanent.

b) "Blue" typhoid: becomes alkaline.

CONSIDERATION OF AGGLUTINATING PROPERTIES OF VARIOUS IMMUNE SERA.

To facilitate interpretation, the results of the experiments have been grouped in the following manner:

I. TYPHOID-IMMUNE SERA.

A. *Typhoid-immune sera which fail to agglutinate typhoid bacilli.*—One of the earliest observations of this nature is by Schumacher, who, with a serum obtained at intervals during an illness of five weeks, failed to agglutinate various strains of typhoid bacilli. The typhoid bacillus was obtained from the spleen of this case post mortem. Similar observations have been made by Jürgens and Ruediger.

On the other hand, mention is also made of typhoid bacilli which failed to agglutinate with typhoid-immune serum. Such, for example, have been noted by Müller, who collected eight cases from the literature and added one of his own; and by Schmidt and Ruediger, each with one observation. In all of these cases the organisms became agglutinable after varying periods of cultivation, Ruediger's B. typh. No. V only during the course of my experiments. Some explanation of these phenomena is offered by Müller, who showed that bacteria grown in the presence of serum of high agglutinative value became less agglutinable; also by Conradi, who found that bacilli from the center of large colonies agglutinated more readily than those from the periphery. Interesting facts analogous to the foregoing developed during the study of B. typh. No. III, obtained from the urine of a typhoid case by Dr. Gehrman. It failed to agglutinate with the serum from three cases of typhoid fever, agglutinated with such sera in seven instances, and with paratyphoid immune sera it agglutinated in much higher dilutions than did other typhoid bacilli. (Tables I, III, V, VI, and VII.)

Immune serum produced by B. typh. No. III agglutinated this organism and paratyphoid bacilli in high dilutions, and typhoid bacilli in low dilutions only. Still more interesting is the fact that these characteristics have persisted for fourteen months, and at the time it was given me it had been cultivated upon artificial

media for several months. Since these peculiarities are in marked contrast to the reactions obtained with other typhoid bacilli, they will not be referred to again.

B. *Typhoid-immune sera with which the typhoid bacillus alone was agglutinated.*—The serum of five cases of typhoid fever possessed this property, as shown by Table I:

TABLE I.

	Case I	Case II	Case III	Case IV	Case V
B. typh. No. VII.....	600	1,000	500	3,000	5,000
B. typh. No. I.....	600	500	200	2,000
B. typh. No. XXX.....	...	50	200	2,000
B. typh. No. V.....	...	500	100	2,000
B. typh. No. XV.....	...	500	200	200
B. paratyph. Buxton (A)...	0	0	0	0	0
B. paratyph. Samuels (B)...	0	0	0	0	0
B. paratyph. Eustis (B)...	...	0	0	0
B. paratyph. No. X (B).....	...	0	0	0	0
B. typh. No. III.....	...	0	0	0

A comparison of the foregoing results with those obtained by other investigators is shown in Table II:

TABLE II.

Authors	B. typh.	B. para-typh. (A)	B. para-typh. (B)	Authors	B. typh.	B. para-typh. (A)	B. para-typh. (B)
Gwyn.....	1,100	0	..	Korte.....	1,280	0	0
.....	90	0	..	Korte.....	80	0	0
Cushing.....	400	0	..	Korte.....	160	0	0
Hume.....	200	0	..	Korte.....	640	0	0
Johnston.....	100	0	0	Korte.....	2,500	0	0
.....	50	0	0	Korte.....	500	0	0
Hewlett, 6 cases..	++	0	0	Case I.....	600	0	0
Longcope, 3 cases.	50	0	0	Case II.....	1,000	0	0
Brion and Kayser.	++	0	..	Case III.....	500	0	0
Allen.....	200	0	0	Case IV.....	3,000	0	0
Korte, 2 cases....	40	0	0	Case V.....	5,000	0	0

In the examination of serum of animals immune to typhoid, to ascertain its power to agglutinate organisms of the typhoid, paratyphoid and colon groups, failure of these sera to agglutinate other than typhoid bacilli has been noted by Hume, Ruediger,

and Smith, each in one case, and by Bruns and Kayser in two cases. Similar properties appearing in the sera of three rabbits are shown in Table III:

TABLE III.

SERA EXAMINED AT VARYING INTERVALS AFTER SUBCUTANEOUS INOCULATION WITH DIFFERENT AMOUNTS OF BOUILLON CULTURE TWENTY-FOUR HOURS OLD.

(Rabbit No. I received 0.5 c.c.; No. II, 2.5 c.c.; No. III, 10 c.c. of B. typh. No. I. Rabbit No. IV received 4 c.c. of B. typh. No. VII.)

Rabbit No.....	Normal			After 3 Days	After 7 Days		After 10 Days				After 18 Days	
	I	II	III	I	I	II	I	II	III	IV	I	II
B. typh. No. VII.	0	0	0	0	4,000	1,000	500	2,000
B. typh. No. I.	50	50	0	50	200	1,000	3,000	3,000	4,000	1,000	1,500	3,000
B. typh. No. XXX.	0	0	4,000	200	750	1,500
B. typh. No. V.	0	0	500	750	2,000
B. typh. No. XV.	0	0	0	80	500	500	1,000	4,000	..	750	1,500
B. paratyph. Buxton (A)	0	0	0	0	0	0	0	0	0	0	0	0
B. paratyph. Sam'ls (B)	0	0	0	0	0	0	0	0	0	0	0	0
B. paratyph. Eustis (B)	0	0	0	0	0	0	0	0	0	0	0	0
B. paratyph. No. X (B)	0	0	0	0	0	0	0	0	0	0	0	0
B. typh. No. III.	0	0	0

In addition to the demonstration of the specific action of the serum, the experiments in Table III illustrate that the agglutinative power of the serum of the first two rabbits was not notably enhanced by increase in the amount of culture employed in single inoculations.

C. Typhoid-immune sera agglutinating both typhoid and paratyphoid bacilli.—Actual agglutination of both typhoid and

TABLE IV.

Case No.	Typhoid	Paratyphoid	
		Group A	Group A
1	320	160	160
2	320	160	160
3	320	40	160
4	320	80	80
5	1,200	40	160
6	1,200	320	320
7	2,500	320	320
8	5,000	320	320
9	5,000	80	320

paratyphoid bacilli with typhoid-immune human or animal serum was obtained by Korte, and Bruns and Kayser. Korte's results were obtained with human serum, and the comparative agglutination values are shown in Table IV.

Of the human sera used in our experiments two were met with which gave similar agglutinative reactions. Their relative inter-agglutinability is shown by Table V:

TABLE V.

	Case I	Five Days Later	Case II
B. typh. No. I	500	500	4,000
B. typh. No. V	500	6,000
B. typh. No. VII	500	6,000
B. typh. No. III	100	1,200
B. paratyph. Buxton (A)	100	0	800
B. paratyph. Samuels (B)	200	50	1,000
B. paratyph. Eustis (B)	150	50	800
B. paratyph. No. X (B)	150	0	1,200
B. coli No. IV	60
B. coli No. X	0

To determine the ability of serum of any given case of typhoid fever to agglutinate other organisms, it would seem necessary to examine it at intervals during the disease, for in Case I (Table V) five days after the first examination agglutinability for the paratyphoid bacilli had almost disappeared.

One instance in which typhoid-immune animal serum manifested these interagglutinating properties is reported by Bruns and Kayser. The serum of a rabbit immunized with the anomalous B. typh. No. III manifested similar properties. B. typh. No. III was agglutinated in a dilution of 1:4,000; other typhoid bacilli, 1:80; paratyphoid bacilli of Groups A and B, 1:2,000; and B. coli No. IV, 1:60.

According to our present conceptions, the power of agglutination possessed by immune serum is due to the presence of certain bodies called agglutinins. These are somatic in origin and depend for their production upon substances contained within the bacterial cell. When two organisms are agglutinated by an immune serum produced by one of them, it is supposed that they contain similar substances, and that their agglutination is in direct ratio to the

amounts of such substances possessed in common. The experiments of Castellani have shown that when such an immune serum is saturated with the immunizing organism, its agglutinating power is lost; whereas, if an excess of other bacteria agglutinated by this serum is added, the serum still retains an agglutinating power for the immunizing organism. On the contrary, when agglutinins are produced by several bacteria, saturation with either does not prevent agglutination of the other. Castellani, de Feyfer, Jürgens, and Korte have used this method to determine the existence or absence of mixed infections. With the demonstration that the serum of Case II (Table V) agglutinated the paratyphoid in such relatively high dilutions the possibility of a mixed infection was at once apparent. To determine this, the growths of B. typh. No. I upon three agar-slant cultures were removed with 50 c.c. of a 1 per cent. solution of NaCl and mixed with an equal quantity of distilled water containing 1 c.c. of the serum. Partial agglutination with precipitation occurred after two hours. The supernatant fluid was removed, filtered, and tested with paratyphoid bacilli. No agglutination occurred in the dilution of 1:200. It was therefore concluded that Case II was not an example of mixed infection, and further proof was afforded by inoculation of bouillon with the patient's blood and the isolation of bacteria from fifteen colonies on a plate made twenty-four hours later; all of these were typhoid bacilli. It cannot be assumed, however, that infection with both organisms might not have existed earlier in the disease.

Whereas, in the preceding account, agglutination was mutually manifested upon the typhoid bacillus and both groups of paratyphoid bacilli, consideration will now be given to sera with which the reaction of the latter organism was restricted to members of one group. The cases in which this selective action was manifested upon members of Group A or B are comparatively few.

D. *Typhoid-immune sera agglutinating typhoid bacilli and members of Group A, but not those of Group B.*—In the table accompanying Korte's work the serum of three cases showed this peculiarity. The dilutions employed were in one case 1:80; in two cases, 1:120. A similar reaction was obtained with the serum

of a rabbit immunized with 4 c.c. of bouillon culture of *B. typh.* No. IV. This bacillus was agglutinated in a dilution of 1:500; *B. paratyph.* Buxton, a member of Group A, in a dilution of 1:100. Four members of Group B. and *B. coli* Nos. IV and X were not agglutinated.

E. Typhoid-immune serum agglutinating typhoid bacilli and members of Group B, but not those of Group A.—Korte met with five cases in which the above reactions were obtained. The dilutions necessary to agglutinate typhoid bacilli were two to fifteen times greater than for the paratyphoid bacilli. In twenty-two cases reported by Jürgens, and in several of our series, typhoid-immune sera were obtained which agglutinated members of paratyphoid Group B, but no tests were made with members of Group A. In some of these cases agglutination with members of Group B took place in greater dilutions than with typhoid bacilli; in some cases it occurred earlier in the disease.

II. PARATYPHOID-IMMUNE SERA.

The recent recognition of paratyphoid fever as a clinical entity has been established largely through the study of isolated cases. In many of these the symptoms simulated those of typhoid fever; in others, the infections were local in character, resembling the sequelae of typhoid. In connection with such diverse clinical manifestations, the exact position of the exciting organism to related groups, its cultural characteristics, and the agglutinative phenomena associated with it have been the subjects of a number of studies. Perhaps the least satisfactory of these have pertained to agglutination. Of the contributions relating to the latter, one of the most important is that of Buxton, who experimented with numerous strains of paratyphoid and related bacilli, and various animal immune sera in arbitrary dilutions of 1:400, and concluded that interagglutination of the various groups does not occur. Careful observations of the agglutinative properties of paratyphoid bacilli made since Buxton's communication have emphasized the necessity of using many dilutions. These observations, together with those of the writer, will be considered as follows:

A. *Sera produced by members of paratyphoid Group A not agglutinating other members of the group.*—Very interesting facts are contained in the history of an organism found by Cushing in the pus of a post-paratyphoidal osteomyelitis. It agglutinated with the patient's serum and that of a rabbit immunized with it. Neither of these sera agglutinated Gwyn's organism, another member of the group. Durham, with an immune serum produced by Gwyn's organism, and Johnston, using the serum from two cases of paratyphoid fever caused by members of Group A, also failed to agglutinate Cushing's organism. One year later Longcope, testing the serum of a case of paratyphoid fever, agglutinated Cushing's bacillus in dilutions of 1:200 and Gwyn's in a dilution of 1:500; and Buxton has since reported that Cushing's organism had acquired agglutinability common to its group. Previously in this article mention has been made of typhoid bacilli which, failing to agglutinate with typhoid-immune serum for a time, have later acquired this property. These instances and the behavior of Cushing's organism would suggest that delayed agglutinability may not prove uncommon.

B. *Sera produced by members of Group A agglutinating members of this group only.*—In four observations—Gwyn, Brion, Kayser, and Hume—the attempts to agglutinate other organisms were limited to typhoid bacilli, and none of these were successful. Bruns and Brion, working with Kayser, were unable to agglutinate typhoid and colon bacilli, or those of Group B, with the serum of a rabbit immunized with a bacillus belonging to Group A. Johnston with three human immune sera agglutinated five members of Group A, as well as one obtained from the blood of one of the cases, but could not agglutinate bacilli of the colon, typhoid, or intermediate groups.

C. *Sera produced by members of Group A agglutinating members of Groups A and B.*—In the experiments with sera immune to typhoid bacilli it was noted that the majority of them exerted a selective action upon members of the typhoid group. The preceding discussion (B) has shown that serum immune to members of paratyphoid Group A possesses similar properties in relation to that group. It therefore has been interesting to ascer-

tain if the latter manifests other properties analogous to those of the typhoid-immune sera which agglutinated members of related groups. Serving to establish this point is a report by Schottmüller of two human immune sera, one of which agglutinated another member of Group A and three members of Group B, the other agglutinated two members of Group B. Smith and Scott, each with the serum of a rabbit immunized with Buxton's bacillus, obtained agglutination with members of both groups. None of these sera agglutinated typhoid or colon bacilli. In my own experiments, agglutination tests were made with a rabbit serum similar to that used by the last two writers, and corresponding results were obtained. B. paratyph. Buxton (A) was agglutinated in a dilution of 1:8,000; B. paratyph. Eustis and No. X (B), 1:6,000; B. paratyph. Samuels (B), 1:4,000. Five strains of typhoid bacilli were not agglutinated.

Previous mention was made that Buxton was unable to agglutinate members of Group B with immune rabbit serum produced by inoculation with his organism. These results show that after several years the same bacillus has acquired the power of producing a serum which readily agglutinates members of Group B.

D. *Sera produced by members of Group A agglutinating members of this group and typhoid bacilli.*—Observations of this character have been made in only one instance. Cushing, with the serum of a rabbit immunized to his organism, obtained agglutination with typhoid bacilli (1:200), and also members of the intermediate group other than paratyphoid.

E. *Sera produced by members of Group A agglutinating members of Groups A and B and typhoid bacilli.*—No cases have been reported in which such an interagglutination was obtained.

Of the observations here recorded we note that in seventeen instances, where agglutination reactions of serum immune to bacilli of Group A were tried with typhoid bacilli, only one positive result was obtained; and in eleven cases, where tests were made with members of Group B, five positive results were obtained. Obviously, these observations are too few to warrant positive conclusions.

The numerous experiments dealing with sera immune to members of Group B will be considered as follows:

F. *Sera produced by members of Group B agglutinating members of this group only.*—Ruediger reported two cases of paratyphoid fever due to members of this group in which the serum did not agglutinate typhoid bacilli. Pratt made a similar observation in one case, and Conradi, Drigalski and Jürgens in altogether four cases. Kayser experimenting with the serum of three cases of paratyphoid fever obtained agglutination with members of Group B, but not with members of Group A or the typhoid group. De Feyfer obtained like results in six cases. Korte with a similar serum could not obtain agglutination with members of the typhoid intermediate or colon groups. Pratt, using the serum of an animal immunized with a member of Group B, could not agglutinate typhoid bacilli.

G. *Sera produced by members of Group B agglutinating members of this group and Group A.*—Instances of this nature are reported by several observers. Schottmüller in three experiments with such sera was able to agglutinate members of Groups A and B, but not bacilli of the typhoid and colon groups. Longcope in three cases, and Allen in two cases, agglutinated members of Group A, but not typhoid bacilli. A similar reaction was observed with animal serum by Scott; with the serum of a rabbit immunized with his paratyphoid organism agglutination was obtained with members of Group A, but not with members of typhoid or colon groups, or other intermediate organisms. I have obtained exactly the same results with this organism.

H. *Immune sera produced by members of paratyphoid Group B agglutinating members of that group and typhoid bacilli.*—Observers reporting reactions of this character are Conradi, Drigalski and Jürgens, in twenty-six cases examined during an epidemic at Saarbrück; Hünemann, in nineteen cases of another epidemic; Lucksch in two cases; and Libman in one case. In two instances, Libman's and one of the Saarbrück series, typhoid bacilli were agglutinated in higher dilutions than were members of Group B, and the possibility of a mixed infection was questioned. In Libman's case, one of cholecystitis, the

diagnosis of a paratyphoid infection was based on the isolation from the blood of a bacillus belonging to Group B. The serum examined later in the disease had lost its power of agglutinating members of Group B, but still agglutinated typhoid bacilli. The diagnosis of the second case mentioned was established by isolation from the stools, of bacilli having characteristics of Group B. In neither case can diagnosis of mixed infection be absolutely excluded.

Jürgens alone and with Conradi and Drigalski obtained, from two rabbits immunized with members of Group B, sera which agglutinated typhoid bacilli. Bruns and Kayser, and Korte, obtained like results with similar immune rabbit's serum; the latter, however, also agglutinated members of colon and intermediate groups.

I. *Immune sera produced by members of Group B agglutinating members of Groups A and B and typhoid bacilli.*—Two human sera have been observed which have manifested agglutination reactions of this character—one by Schottmüller, and another by Korte. That observed by the latter also agglutinated other members of the intermediate group. No agglutination with colon bacilli was obtained. Korte, with the serum of two immune rabbits, was able to agglutinate members not only of Groups A

TABLE VI.

IMMUNE RABBIT SERA OBTAINED FOURTEEN DAYS AFTER SUBCUTANEOUS INOCULATION WITH 4 C.C. OF TWENTY-FOUR HOUR BOUILLON CULTURES OF BAC. PARATYPH. SMITH, EUSTIS, AND NO. X, GROUP B.

	Smith	Eustis	No. X
B. paratyph. Buxton (A)	200	500	6 000
B. paratyph. Samuels (B)	500	8,000
B. paratyph. No. X (B)	500	6,000
B. paratyph. Smith (B)	200
B. paratyph. Eustis (B)	500	200	6,000
B. paratyph. Scott (B)	200
B. typh. No. III	0	200	6,000
B. typh. No. I	50	50
B. typh. No. V	50	50	0
B. typh. No. XV	50	50	0
B. typh. No. XXX	0	50	50
B. coli No. IV	50
B. coli No. X	0

and B and the typhoid group, but also of the colon and intermediate groups. In our series of experiments with rabbits immunized with members of Group B, three sera were obtained which agglutinated members of Groups A and B and typhoid bacilli. These results are shown in Table VI.

III. COLON AND OTHER IMMUNE SERA.

It has been observed that certain of the typhoid- and paratyphoid-immune sera have agglutinated members of the colon and intermediate groups, and it is important to ascertain whether sera immune to members of these groups will agglutinate typhoid and paratyphoid bacilli. Exhaustive contributions have been made in regard to the action of these sera on typhoid bacilli and the experiments of Castellani, Durham, Bruns and Kayser, Stern, Biberstein, Rothberger, and Theobald Smith demonstrate that typhoid bacilli are occasionally agglutinated with serum produced by members of the colon and intermediate groups.

The experiments relative to the action of these sera on paratyphoid bacilli are less numerous. Scott failed to agglutinate his member of Group B with the serum of two rabbits immunized with *B. enteritidis* and *B. dysenteriae*, respectively. Hume reports the failure of members of Group A to agglutinate with the serum of a rabbit immune to *B. enteritidis*. Cushing, however, obtained a serum from a rabbit immunized with *B. chol. suis*, which did agglutinate a member of Group A. G. B. Smith, with the serum of two rabbits immune to *B. chol. suis* and *B. enteritidis*, failed to obtain agglutination with members of either group.

Three reports—Hume, Bruns and Kayser, and Scott—concern the action of serum immune to members of the colon group on paratyphoid bacilli, and in none of these did agglutination occur.

Table VII shows the results obtained with serum immune to the two colon bacilli used in our experiments. The organism *B. coli* No. IV, which at times agglutinated with paratyphoid- and typhoid-immune sera, produced a serum which agglutinated these bacilli, whereas serum immune to *B. coli* No. X did not acquire this property.

TABLE VII.

COLON-IMMUNE RABBIT SERA OBTAINED FOURTEEN DAYS AFTER SUBCUTANEOUS INOCULATION WITH 4 C.C. TWENTY-FOUR HOUR BOUILLON CULTURE OF *B. COLI* NOS. IV AND X.

	<i>B. coli</i> No. IV	<i>B. coli</i> No. X
<i>B. coli</i> No. IV	1,000	500
<i>B. coli</i> No. X	0	0
<i>B. paratyph.</i> Buxton (A) ..	60	0
<i>B. paratyph.</i> Eustis (B) ...	80	0
<i>B. paratyph.</i> Samuels (B) ..	60	0
<i>B. paratyph.</i> No. X (B)	80	0
<i>B. typh.</i> No. I	60	0
<i>B. typh.</i> No. V	40	0
<i>B. typh.</i> No. XV	40	0
<i>B. typh.</i> No. XXX	0	0
<i>B. typh.</i> No. III	150	0

Further experiments of this character will probably show that paratyphoid bacilli interagglutinate with colon and other immune serum to the same, or even a greater, extent than do typhoid bacilli.

SUMMARY.

A review of these results would suggest that certain phases of this subject require still further investigation before the question of interagglutination of bacilli of the typhoid and paratyphoid group can be placed on a reliable basis. Relative to this fact should be mentioned that a number of the paratyphoid bacilli reported have occasionally manifested cultural reactions which differed from those characterizing the group in which they have been placed. Some of these irregularities may be explained by different environments, or variations in the culture media used by the various observers, but they nevertheless constitute evidence that hard and fast lines cannot be established between the typhoid, paratyphoid, and other intermediate groups.

If, in the light of our present knowledge, a precise classification of these bacilli into groups cannot be arranged, we should not expect to make definite conclusions in regard to the interagglutination of members of these groups. It must therefore be acknowledged that the most reliable means of diagnosis is the study not only of the exciting organism isolated from the blood

or feces with agglutination tests, but also of cultural characteristics, for it has been shown that the diagnosis of bacilli by the agglutination tests, as established by several investigators reporting results of blood cultures, is untrustworthy.

The difficulties encountered in the isolation of the causative bacillus, and the impossibility of isolation in the later stages of the disease have led, as before stated, to the use of agglutination reaction as a convenient and presumably accurate test. The results of these experiments have not established the inaccuracy of this procedure, as would at first appear, but have emphasized the necessity of modifying the prevailing interpretations of the occurrence or non-occurrence of the reaction.

It has been assumed that agglutination of typhoid bacilli by a suspected serum in a dilution of 1:100 by the microscopic method, or of 1:50 by the macroscopic method, is an indication of infection with this organism. However, as shown in the foregoing, serum immune to members of other groups may also agglutinate the typhoid bacillus in the same, and even higher, dilutions, and a test with such bacilli, especially those of paratyphoid Groups A and B, should be performed. Ordinarily the paratyphoid organisms require from ten to thirty times more serum for their agglutination than do typhoid bacilli, and if reactions of the suspected serum with these occur in relatively high dilutions, the saturation test is necessary to decide which is the organism producing the infection.

If repeated agglutination tests made at intervals in a suspected case prove negative with both typhoid and paratyphoid bacilli, it either denotes infection with some other organism, or possibly may be analogous to the reported cases in which typhoid bacilli were isolated from the blood or stools, and no agglutination of other typhoid bacilli occurred with the patient's serum.

In suspected cases of paratyphoid fever, agglutination of members of both groups should be tried, as serum immune to members of one may not agglutinate members of the other group. In other instances serum immune to members of either group may agglutinate the typhoid bacillus in high dilutions, and here again the saturation test is necessary. Some of these difficulties may

be avoided by using for the tests bacilli which are known to agglutinate only with serum immune to members of their own group. In the event that representatives of the various groups are not at hand, diagnosis by means of the agglutination test may be exceedingly difficult. It is to be hoped that in the near future a technic will be perfected by which will be obtained reactions which are sufficiently delicate and specific to restore the agglutination test to a comparatively simple procedure.

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THE BACTERIAL PRECIPITINS.*

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INTRODUCTORY.

A LONG series of complicated studies upon the body fluids made during the past few years has led to the discovery of a number of hitherto unknown or little understood substances which seem to be of great significance in the processes of immunization of man and the lower animals to bacteria or to their toxins, and in the adaptation of the body to a great variety of alien materials. Many of these substances are present in the blood serum under normal conditions, and they may be increased in amount, or entirely new substances, it seems, may be formed when the body adapts itself to foreign material artificially introduced into the veins, subcutaneous tissue, or the peritoneal cavity, or after adap-

tation following upon the course of a bacterial infection or disease. The substances, whether normally present in serum or developed as a result of adaptation, are commonly designated "antibodies," and the serum containing them is known as an "antiserum." For example, the serum of a normal rabbit may contain a substance which agglutinates the typhoid bacillus—an "agglutinin" specific within certain limits for this bacillus. The normal agglutinating substances are, however, greatly increased in amount or in their activity by the adaptation of the rabbit to the typhoid bacillus or to its metabolic products artificially introduced into the body of the animal; or, if the agglutinin be not normally present, it may be formed *de novo* in response to the process of artificial adaptation. The agglutinin is an example of an antibody, and the serum in which it is developed is called an antityphoid serum.

Besides bacteria and their products, various animal or vegetable substances, generally albuminous in nature, such as alien blood sera, serous fluids, or their isolated albumins and globulins, as well as milk, peptone (?), egg albumin, various ferments, and the cells of various organs, or their extracts, derived from different animals, induce, after introduction into the animal organism, the formation of antibodies. These are of several kinds; thus, specific antitoxic, bactericidal, cellucidal, agglutinating, and precipitating properties have been demonstrated in sera.

As a result of the adaptation of animals to serum containing these antibodies, a second series of substances may in many instances be developed which neutralize or render inactive these antibodies. In this manner, antiagglutinins, antiprecipitins, anticoagulins, antiamboceptors (antilyns), etc., are said to be formed.

Rudolf Kraus,¹ April 30, 1897, described a class of hitherto undiscovered substances in the sera of animals immunized or adapted to certain species of bacteria. Kraus found that cholera, plague, and typhoid antisera, when added to their corresponding bacterial filtrates, cause precipitates. The substance of the serum which causes the precipitation was called a precipitin. On adding an antiserum, say of typhoid, to the heterologous filtrates of plague or cholera, for example, no such reaction, however, took place. The reaction between these antisera and their correspond-

ing filtrates is thus, according to him, strictly specific, and hence as diagnostic as the Gruber-Durham reaction of agglutination was at that time considered to be. This view for the bacterial precipitins has never been seriously questioned,* although A. Wassermann² observes that, as in the case of the agglutinins, bacteria belonging to species closely related possess similar precipitin receptors, indicating that agglutination and precipitation are not strictly specific phenomena. Recently Tupnix,³ in an abstract, has indicated that bacterial precipitins are by no means as specific as one might infer from the articles which had up till then appeared. A few other references of similar import are found in the literature, but they need not be cited.

On the other hand, the researches on non-bacterial precipitins have clearly shown that the precipitins which are developed in an animal of one species after adaptation to the serum of another species are not specific. These hemo- or sero-precipitins, as they are called, thus precipitate, not only the serum of the species of animal by the use of which they have been developed, but also the sera of species more or less closely related. They are, however, markedly special, for a precipitin invariably gives a quicker and more copious reaction in the homologous than in a heterologous serum.

The general analogy existing between the action of the bacterial precipitins and sero-precipitins led the writer to believe that a more careful study than had hitherto been attempted might reveal relationships between the bacterial precipitins similar to those existing between the sero-precipitins, as mentioned above.

Furthermore, the constant association of the bacterial agglutinins and precipitins in antisera, and the striking points of similarity in the action of these two antibodies, seemed to indicate that between the bacterial precipitins relationships would be demonstrated similar to those which had been conclusively shown to exist between the bacterial agglutinins at the time these studies were begun—in the fall of 1901.

Before undertaking the main part of our task upon the inter-

* The term "bacterial precipitin" is used by the writer to indicate the substance in the serum of normal or adapted animals which induces the precipitation of bacterial broths, filtrates, or other culture extracts.

relations of the bacterial precipitins, a series of observations were made to determine the precipitating action of normal rabbit serum upon various bacterial filtrates.

Although bacterial precipitins have not hitherto been demonstrated in normal sera, the fact that they have been found in association with the agglutinins in the serum of animals adapted to different bacterial species—so constantly indeed that some observers consider these antibodies identical substances—seems to indicate that their presence in normal sera may either have escaped attention, or that, if present, they are in quantities so small that their detection is not possible.*

On the other hand, the possibility of precipitates forming in mixtures of such complex fluids as nutrient broth and serum, by some action other than that induced by normal precipitins, cannot be summarily dismissed.

These reasons led the writer to investigate the precipitating action of normal and various antibacterial sera upon plain nutrient broth and upon various bacterial filtrates.† The tests which are now to be described are preliminary, and serve as controls for the subsequent series described later on.

*The precipitins, however, unlike the agglutinins, are infrequently found in appreciable quantities in normal serum. According to Linossier and Lemoine,⁴ the hemo- or sero-precipitins of one species for the serum of other species of warm-blooded animals do not exist in the serum of warm-blooded animals. Noguchi⁵ has, however, found normal precipitins in horse serum, for the serum of some species of cold-blooded animals, and also that "the sera of some cold-blooded animals contain precipitins." Noguchi states: "It might be said that the existence of normal precipitins for a given serum is more likely to occur in a widely than a closely related species." He assumes that the relationship between the natural precipitins and those produced by adaptation will be found analogous to that which exists between the corresponding natural and artificial agglutinins and hemolysins.

Many observers have noted the fact that the development of adaptation precipitins for the serum of all species is more readily induced in the organism of a species not too closely related. Rabbit serum, according to Lamb,⁶ contains normally a precipitin for cobra venom, and also, according to Obermayer and Pick,⁷ for the dysglobulin of the whites of eggs.

†The culture filtrates which were employed during the course of our investigations, when not otherwise so stated, were obtained from broth made up with Liebig's beef extract, 5 g., 1 per cent. peptone, and 0.5 per cent. sodium chloride, the reaction being approximately 0.5-1 per cent. acid to phenolphthalein. The broth cultures were passed through Berkefeld filters, after an incubation of several months at 37° C. The reaction of our filtrates was alkaline to litmus paper, except that of the diphtheria filtrate, which was acid. The precipitation and agglutination tests were made in sterilized tubes of approximately the same size, and of narrow caliber.

The macroscopic agglutination tests described in this paper were made with suspensions of eighteen-hour agar cultures in 10 c.c. of sterile normal saline solution. After settling for half an hour, 0.9 c.c. of the emulsion, 0.1 c.c. of the serum, or of its dilutions in normal saline solution, was introduced into each tube. Controls were always made.

II. THE PRECIPITATING ACTION OF NORMAL AND ANTIBACTERIAL SERA ON BROTHS OF VARIOUS REACTIONS AND UPON VARIOUS BACTERIAL BROTH FILTRATES.

Normal rabbit and ox serum, as well as antibacterial rabbit sera, were added to broths of various composition and reactions. These broths had a basic composition of 5 g. Liebig's meat extract, 1 per cent. Witte's peptone, 0.5 per cent. NaCl; but varied in their reaction, as follows: 1 per cent. and 0.5 per cent. alkaline, neutral; 0.5 per cent., 1 per cent., and 1.5 per cent. acid, phenolphthalein being the indicator, or were made from the usual meat infusion of different reactions, or of broth made up without additions of pepton.

The tests were made with the following proportions: to 0.5 c.c. of broth was added 0.1 c.c., 0.25 c.c., and 0.5 c.c. of serum. The tests showed that when normal rabbit serum is added to the meat extract broth of 1.5 per cent. acid reaction, small flocculi may develop after twenty-four hours at 37° C. In most cases, however, there is merely a fine granular deposit, which develops on the sides of the tube and is clearly seen only with a low power lens, the fluid remaining clear. This precipitation was never seen in broth of other reactions. In the usual meat infusion, pepton salt broth, normal rabbit serum produces no granular precipitate or flocculi. With normal ox serum no precipitation occurs in broths of different reactions. Normal rabbit and ox serum give rise to opalescence and later to cloudiness, with or without the formation of granules and flocculent precipitates, in broth made up without the usual addition of pepton or salt. The most marked reactions are obtained in broth of 1 per cent. or 1.5 per cent. acid reaction.

In 4 per cent. acid broth a marked turbidity is constantly developed. The reactions obtained in alkaline broth are slight. The nature of the slight precipitates was not determined; in some cases they were possibly small coagula, due to late or uncompleted clotting of the sera, or to the deposition of the cellular elements contained in the sera. Since the reaction of our filtrates was usually alkaline, the cloudiness and precipitates that are developed in broth of 0.4 or 1.5 per cent. acid reaction can be disregarded in our subsequent tests.

The insignificant and late precipitates which are occasionally formed may be disregarded, since they cannot be mistaken for the precipitates developed by the bacterial precipitins upon broth filtrates. The numerous tests made show conclusively that normal rabbit and ox serum, as well as various antibacterial rabbit sera, exert no precipitating action upon the usual peptone salt broth of slight alkaline or acid reaction.

Of especial importance was the observation that normal rabbit serum does not precipitate the various bacterial broth filtrates,* which for the sake

* The broth filtrates of the following species of the colon-typhoid group were tested: *B. typhosus*, *B. coli* "Escherich," *B. psittacosis*, *B. typhi murium*, *B. enteritidis*, *B. ieteroides*, *B. Schottmüller* (Seemann and Müller), *B. Gwyn*, *B. Cushing*, and *B. No. 1*, a species of the intermediate group of Durham. The species of the Dysentery group tested were the *B. dysenteriae* Kruse, *B. dysenteriae* "New Haven" (Duval), *B. dysenteriae* "Y" (Hiss). The filtrates of the following bacteria were also tested: *B. proteus*, *B. prodigiosus*, *B. pyocyaneus*, *B. tuberculosis*, human and bovine, *B. diphtheriae*, *Sp. cholera Asiaticae*, *Sp. Metchnikovi*, *Staphylococcus pyogenes aureus*, *Pneumococcus*, and *Streptococcus pyogenes*.

of brevity need not be enumerated here. We are therefore warranted in drawing the conclusion that normal rabbit serum contains no determinable amount of normal bacterial precipitins. The few specimens of ox serum examined likewise indicate the absence of bacterial precipitins in this serum. The question of the presence or absence of normal bacterial precipitins in the serum of other warm- or cold-blooded animals was not determined.

Having demonstrated the absence* of determinable amounts of precipitating substances in the serum of normal rabbits for the filtrates of the different bacterial species which were employed during the course of these studies—an observation in complete accord with those of previous investigators—the writer now purposes to describe a series of observations on the precipitating action of a number of antibacterial sera upon the filtrates of their corresponding and of heterologous species of more or less closely related bacteria: in other words, to test the specificity of the precipitins.

III. THE PRECIPITATING ACTION OF THE SERA OF RABBITS ADAPTED TO VARIOUS SPECIES OF THE COLON-TYPHOID GROUP IN THEIR RESPECTIVE CULTURE FILTRATES.

GENERAL PLAN OF STUDY.

The plan followed in these researches was, first, to study the precipitating action of the serum of rabbits adapted to several distinct species of the so-called colon-typhoid group, in order to determine whether the precipitative relationships exist between various species of this group; and, secondly, to search for more remote relationships, as indicated by slight and late reactions between the bacilli of this and those belonging to other groups of bacilli—namely, *B. proteus*, *B. prodigiosus*—as well as those

* In a strict sense the absence of precipitin haptins for bacteria in normal serum cannot be determined positively, for they may be present in quantities so slight as to escape detection. The writer's observations convince him that the precipitins and agglutinins in antibacterial serum, as a rule, bear the relative proportion, roughly speaking, of 1-100 and that, as a rule, the agglutinin must react in a dilution of 1-100 "macroscopic test" before the presence of a precipitin can be certainly detected. The fact that normal rabbit serum rarely agglutinates bacterial species above 1-100 accounts sufficiently, if the above argument is correct, for the non-detection or absence of the normal precipitins. The writer believes, as will be described later, that this fact cannot be regarded as proof of the identity of those two substances, for an antiserum may precipitate the filtrate of a given species of bacterium without agglutinating the bacterium; and he believes, furthermore, that the precipitins may be formed for a given species without giving rise to any, or only agglutinins capable of reacting in low dilutions.

bacteria belonging to other classes of bacteria, the spirilla and the cocci.

These introductory remarks suffice to indicate the scope of the writer's studies. The first part of our investigations will now be described:

The species used in these series of tests were, first, a bacillus belonging to the hog cholera or intermediate group of Durham, marked B. No. 1; second, a collection culture of *B. typhosus*, designated *B. typh.* "Coll.:" and, third, a colon bacillus which does not ferment saccharose.*

Sets of rabbits were adapted to each of these species, and the antisera thus obtained were tested, as far as practicable, at the same time, so that their precipitating action upon the same filtrates could be carefully compared. For convenience the results obtained will be described in series.

SERIES I.

ANTI-B. NO. 1 RABBIT SERUM.

Rabbit I received 14 c.c. of heated and unheated broth cultures at intervals of several days (November 9, 1901, to January 1, 1902). Bled five days later. The serum agglutinated *B. No. 1* up to 1-10,000 (microscopic test); the *B. typh.* "Coll." to 1-2,000 (microscopic test).

The serum was tested upon the filtrates of the three species which had been grown in broth at 37° C. for twenty-nine days. The filtrates were markedly alkaline to litmus.

TABLE I.

THE PRECIPITATING ACTION OF THE ANTI-B. NO. 1 SERUM UPON ITS CORRESPONDING FILTRATE AND UPON THE FILTRATES OF *B. TYPHOSUS* AND *B. COLI*.

B. No. 1 filtrate: 1 c.c.	Anti-B. No. 1 serum: 0.5 c.c.	Tubes kept at 37° C. 2-3 hrs. Cloudiness and flocculent precipitates which soon settle.
<i>B. typh.</i> filtrate: 1 c.c.	Anti-B. No. 1 serum: 0.5 c.c.	Cloudiness is slightly delayed when compared with the homologous filtrate, but at 5 hrs. the reaction is only somewhat less marked. Same phases of reaction.
<i>B. coli</i> filtrate: 1 c.c.	Anti-B. No. 1 serum: 0.5 c.c.	Same phenomenon, only onset of reaction is slower, and the precipitum formed is less than in case of typhoid filtrate.

Tests were then made with similar quantities of the filtrates, but with 0.1 c.c. of serum. With *B. No. 1* filtrate the various phases of reaction occurred as rapidly, the precipitum being, however, less abundant; with the typhoid filtrate the reaction was slightly delayed when compared with that

* *B. No. 1* and the colon bacillus were isolated from the organs of a patient dying from an infection of five days' duration, clinically a case of severe gastro-enteritis with toxemia.

in the homologous filtrate; a similar, but less marked, reaction was obtained with the colon filtrate.*

To avoid the criticism that we were possibly not dealing with a true typhoid bacillus, the B. No. 1 serum was tested, with identical results, against a filtrate of the well-known typhoid bacillus isolated by Pfeiffer, and known as the "Pfeiffer" culture. On account of the saprophytic condition of the "Pfeiffer" culture, which was only slightly motile, and the rapid pseudo-clumping of its broth cultures and agar suspensions in controls, the agglutinative value of the serum for this bacillus could not be determined.

We thus see from our experiments that the anti-B. No. 1 serum precipitated in dilutions of 1-3 and 1-10, not only the corresponding, but the heterologous, typhoid and colon filtrates; the onset of the reaction cloudiness, however, developing earliest in the homologous filtrate. The amount of precipitum ultimately formed in the B. No. 1 and typhoid filtrates was apparently similar.

ANTITYPHOID RABBIT SERUM.

At intervals of three to six days Rabbit II received subcutaneously ten inoculations of broth cultures, at first heated and then unheated, in all about 25 c.c. Agglutination value, 1-10,000, complete in three hours by macroscopic test; 1-5,000, complete in one hour. The serum did not agglutinate B. No. 1 or the B. coli.

TABLE II.

THE PRECIPITATING ACTION OF THE ANTITYPHOID SERUM UPON ITS CORRESPONDING FILTRATE AND UPON THE FILTRATES OF B. NO. 1 AND B. COLI.

B. typh. filtrate (Coll.): 1 c.c.	Antityph. serum: 0.5 c.c.	At 37° C.: 1 hr., cloudiness; 2 hrs., flocculi; and later, copious precipitate.
B. No. 1 filtrate: 1 c.c.	Antityph. serum: 0.5 c.c.	At 37° C., 2 hrs., cloudiness, flocculi; and later, copious precipitate.
B. coli filtrate: 1 c.c.	Antityph. serum: 0.5 c.c.	No reaction at 2 or 4 hrs.; at 18 hrs., small precipitate.

In a filtrate of a 1 per cent. mannit-broth typhoid culture grown for two weeks at 37° C., having a terminal acid reaction, the onset of the reaction was delayed to twelve hours, and the precipitum formed was less copious than that formed in the B. No. 1 filtrate. With the anti-B. No. 1 serum, this filtrate likewise gave a delayed reaction.

These experiments show, therefore, that the antityphoid serum precipitates its corresponding filtrate and the B. No. 1 filtrate in

*In judging the presence or absence of slight precipitates, to secure accuracy it is imperative to examine the deposit for bacteria; for non-cloudiness in serum mixtures does not signify absence of bacterial growth. When in doubt, tests were made by culture and by examination of coverslips. Usually there is no difficulty in distinguishing between precipitates and bacterial bottom growths, especially if the development of the reaction is watched.

the dilution of 1-3 and 1-5; the reaction being quicker, however, but not more copious, in the homologous filtrate obtained from a broth culture of the same age and terminal reaction. The reaction in the colon filtrate was considerably delayed. That the age and perhaps the terminal reaction of the filtrate exert considerable influence upon the time of onset of the precipitation and the amount of precipitum is shown by the observation that the anti-B. No. 1 serum gives rise to a quicker and more copious reaction in the alkaline typhoid filtrate (four weeks' broth culture at 37° C.) than does the antityphoid serum in the acid typhoid filtrate obtained from a two weeks' mannit-broth culture.

ANTICOLI RABBIT SERUM.

Rabbit III, at intervals of three to five days, received subcutaneous inoculations of heated and unheated broth cultures, in all 24 c.c., one to four days' growth at 37° C. (January 3 to February 10, 1902). Bled several days after last injection. No agglutination of the homologous colon bacillus either by the macroscopic method or by the hanging drop. A slight tendency to thread formation was observed, which ceased in a dilution of 1-1,000. With a serum dilution of 1-10, complete settling occurred only after twenty-four hours at 37° C.

TABLE III.

THE PRECIPITATING ACTION OF THE ANTICOLI SERUM UPON ITS CORRESPONDING FILTRATES AND UPON THE FILTRATES OF B. NO. 1 AND B. TYPHOSUS.

Coli filtrate:	Anticoli serum:	Cloudiness in all tubes in 1 hr. at 37° C., which increases rapidly. 6 hrs., well-marked precipitum, most copious in tube containing 0.25 c.c. serum. The fluid in all the tubes becomes clear.
0.5 c.c.	0.05 c.c.	
0.5	0.10	
0.5	0.15	
0.5	0.25	
B. No. 1 filtrate:	Anticoli serum:	At 6 hrs., a slight precipitate in the form of fine granules has appeared on the sides of the tubes. At 24 hrs. in the tube containing 0.25 c.c. of serum the precipitum compares in amount to that formed in the homologous filtrate 0.05 c.c. of serum.
0.5 c.c.	0.10 c.c.	
0.5	0.25	
B. typh. Coll. filtrate:	Anticoli serum:	Similar reactions to that obtained in B. No. 1 filtrate.
0.5 c.c.	0.10 c.c.	
0.5	0.25	Similar delay in reaction to that obtained with Typh. Coll. filtrate.
B. typh. Pfeiffer fil.:	Anticoli serum:	
0.5 c.c.	0.1 c.c.	
0.5	0.25	

Thus the anticoli serum in dilutions of 1-6 precipitated the corresponding coli filtrate, and also the typhoid and B. No. 1 filtrates. The onset of the reaction in the heterologous filtrates was, however, delayed, and the amount of precipitum formed was considerably smaller than in the colon filtrate.

SUMMARY OF SERIES I.

The results of the first series of tests may be briefly summarized. Precipitative relationships exist between three species of

the colon typhoid group; namely, the intermediate *B. No. 1*, the *B. typhosus*, and the *B. coli*, micro-organisms distinguished from each other by constant biochemical characters of differential value.

ARE PRECIPITINS DEVELOPED IN THE SERUMS OF RABBITS WHICH HAVE ADAPTED THEMSELVES TO INJECTIONS OF PEPTONE?

The rabbits of Series I described above were adapted by means of the usual peptone-broth cultures. Myers,⁸ however, had noted the fact that Witte's peptone gave rise to the formation of substances in the serum of the animals undergoing adaptation which precipitated peptone solutions. This observation led us to suspect that the precipitative relationships noted above between filtrates of various bacterial species, distinct at least as to their biochemical characters, were possibly due alone to the development of a peptone precipitin in the sera of the rabbits of Series I which had been inoculated, as mentioned above, with peptone-broth cultures.

To remove all doubt upon this score, the writer was led to repeat Myers's observations, in order to test his statement that the inoculation of peptone in the animal organism is followed by the formation of substances which precipitate peptone solutions. Our experiments and the literature bearing upon this topic may be briefly reviewed.

Two rabbits were treated with 108 c.c. of a (Witte's) 4 per cent. peptone solution, six intraperitoneal inoculations being made at intervals during four weeks. The sera obtained did not cause even an opalescence in fresh peptone solutions, when added either in equal volumes or in the proportion of 1-5, after twenty-four hours at 37° C. A rabbit treated with large quantities of broth likewise failed to develop precipitating agents in its serum. Intravenous inoculations (five to six inoculations of 0.5 g. of Witte's and Merck's peptone) have likewise proved negative in the hands of my colleagues, Drs. Wood and Jessup, to whom I am indebted for the information.

Myers's observations, although widely quoted and accepted, have likewise not been confirmed by several other investigators. He claims to have obtained precipitins in the serum of rabbits that had adapted themselves to solutions of Witte's peptone. In an article published previous to Myers's, Tchistowitch⁹ states that he had failed to obtain peptone precipitins in the serum of rabbits adapted to peptone. Buchner and Geret¹⁰ cast grave doubts upon the nature of the peptone precipitum obtained by Myers, since it gave no biuret reaction, and, furthermore, because the anti-peptone serum after inactivation by heat was reactivated by normal serum. They endeavored to obtain peptone precipitins by adapting rabbits to peptone, prepared according to Kuhne's method. The rabbits yielded a serum which, when added to a 2 per cent. peptone normal saline solution, gave rise to cloudiness and a fine precipitate composed of so-called globulites, which were found to be crystals of barium sulphate of unusual form.

Rabbits treated with a single injection of 5 c.c. of ox serum after twenty-four hours likewise yielded a serum which developed these crystals.

These observers found that the peptone of commerce invariably contained barium salts. The solutions of peptone freed from the barium salts

were, however, not precipitated by the sera of the rabbits which had been adapted to peptone or to serum.

Michaelis also failed to obtain precipitins in the serum of rabbits which had been treated for six weeks with subcutaneous inoculations of a 10 per cent. solution of Merck's egg peptone (albumose): the receptors which in the whole albumin molecule induce precipitin formation being, Michaelis believes, destroyed by the pepsin digestion to which the egg albumin has been subjected. Similarly with "Peptonum siccum Riedel," which is a mixture of the proteoses derived from the fibrin of ox serum, no precipitating serum was obtained. Although Kowarski obtained precipitins for the albumoses of seeds, Michaelis considers that these albumoses are not comparable to the pepsin albumoses of animal origin, and that, even if in truth albumose and peptone precipitins can be formed, the receptors which would induce the formation of the egg peptone antibodies cannot be identical to those of egg albumin, for an egg albumin precipitin does not precipitate egg-peptone solutions.¹¹

Obermeyer and Pick¹² have likewise failed to obtain peptone precipitins, although they noted occasionally slight clouding of the peptone solutions by antipeptone serum. That the question is not finally settled is indicated by the recent investigation of Rostoski and Sacconaghi,¹³ who report that after several failures they succeeded in obtaining antibodies or precipitins in the serum of rabbits which had been inoculated with the peptic and tryptic cleavage products, of horse serum albumin. The sera precipitated the solutions of albumin and those of all the cleavage products, considerable quantitative differences being observed in the reaction, the weakest being obtained in peptone solutions.

Rostoski assumes that the immunizing body, whether it be a special side molecule of the giant albumin molecule, or a body which is merely intimately attached to the albumin molecule, is variously resistant to digestion, according to its association with the different cleavage products. In some cases it is more resistant than the albumin body itself, especially to the action of trypsin.

Ascoli has observed that antisera precipitate the cleavage products of albumin. Thus antihorse and antiox serum of the rabbit precipitate in a slight degree Witte's peptone.* He assumes that digestion destroys some of the groups of the albumin molecule (side chains or receptors of Ehrlich), whereas other groups, those genetically related to the peptone molecule, are left unaffected.

P. A. Levene¹⁴ claims to have shown that a precipitin active for a given proteid is capable of forming precipitates with the primary digestion products of these proteids.

A further proof that the precipitative relationships which had been shown to exist were not due to the formation of precipitins for peptone was furnished by the fact that the antisera of Series I do not precipitate our controls of plain peptone broth, or even 1 per cent. peptone solutions. Nevertheless, to remove all possibility of error, all subsequent rabbits "in this series of studies" were inoculated with emulsions of agar cultures, and not with peptone-broth cultures.

* Possibly this is accounted for by the globulite reaction of Buchner and Geret.

SERIES II.

The tests with the antisera of animals of the first series adapted to the three species above mentioned were preliminary. Those made with the sera of the rabbits of Series II, adapted to the same species, are now given, so far as possible in tabulated form, for brevity of presentation. The rabbits of this series, as well as all subsequent animals, were adapted by inoculations of salt emulsions of agar cultures, grown for twenty-four hours at 37° C. The early inoculations were made with heated and the later with living cultures.

ANTITYPHOID RABBIT SERUM.

Rabbit IV received several inoculations.* Agglutination limit of its serum was found to be 1-1,000 for *B.* typh. "Coll." No action on *B.* No. 1 or *B. coli*.

TABLE IV.

THE PRECIPITATING ACTION OF THE ANTITYPHOID SERUM UPON ITS CORRESPONDING FILTRATE AND UPON THE FILTRATES OF *B.* No. 1 AND *B. COLI* AND OF VARIOUS OTHER SPECIES OF THE COLON-TYPHOID GROUP.

Typh. fil. (Coll.):† 0.5 c.c.	Antityp. serum 0.1 c.c.	At 2 hrs. at 37°C.: cloudiness.	At 7 hrs.: pre- cipitation has settled out.	At 19 hrs.: scanty precipitum.
0.5	0.25	Fine flocculent precipitate.	At 7 hrs.: pre- cipitation has settled out.	Moderate amount of precipitate.
<i>B. No. 1</i> fil.: 0.5 c.c.	0.1	Negative.	Negative.	Negative.
0.5	0.25	Negative.	Negative.	Distinct flocculent precipitate.
<i>B. coli</i> fil.: 0.5 c.c.	0.1	Negative.	Negative.	Small flocculi.
0.5	0.25	Negative.	Negative.	Precipitum.
<i>B. icteroides</i> fil.:‡ 0.5 c.c.	0.25	At 19 hrs.: nega- tive.	At 43 hrs.: slight cloudiness?	
<i>B. psittacosis</i> fil.: 0.5 c.c.	0.1	Slight precipi- tation.		
0.5	0.25	Abundant pre- cipitum.		
<i>B. typhi murium</i> fil.: 0.5 c.c.	0.1	At 2 hrs.: nega- tive.	At 19 hrs.: small flocculi.	
0.5	0.25	Slight cloudi- ness.	At 19 hrs.: small precipitate.	
<i>B. dysen.</i> Kruse fil.§ <i>B. dysen.</i> Flexner fil.: 0.5 c.c.	0.25		Small precipitate.	
<i>B. enteritidis</i> : 0.5 c.c.	0.1	At 2 hrs.: nega- tive.	At 7 hrs.: nega- tive.	At 19 hrs.: negative.
0.5	0.25	At 2 hrs.: nega- tive.	At 7 hrs.: nega- tive.	Small flocculi.

* Rabbit IV developed snuffles during adaptation.

† The typhoid, colon, and *B. No. 1* filtrates in these and the subsequent tests were obtained from meat-extract broth cultures grown at 37° C. for ninety-seven days.

‡ The following broth filtrates of same length of incubation at 37° C. were also tested.

§ We are indebted to Professor Flexner for these cultures, which do not ferment mannite, and therefore belong to the true Shiga type, incorrectly called "alkaline" type of *B. dysenteriae*.

A staphylococcus filtrate gave no precipitate after forty-three hours.

It has been shown that, although the agglutinating and precipitating action of the antityphoid serum was considerably weaker than that of the typhoid antiserum of the first series, nevertheless the serum precipitated the filtrates of all the colon-typhoid group tested, the negative result obtained with the filtrate *B. icteroides* being most probably due to the scanty growth of the bacillus in the broth from which the filtrate was obtained.

ANTI-B. NO. 1 SERUM.

Rabbit V between March 19 and April 2, 1902, received five agar cultures; bled April 18. The following microscopic agglutination tests were made with the serum upon B. No. 1:

1-20,000: nearly complete in $\frac{3}{4}$ hr.; complete in 3 hrs. at 37° C.; and 1-40,000, complete at 6 hrs.

The serum tested upon the typhoid bacillus gave the following reaction:

1-100: at $\frac{1}{2}$ hr. reaction begins, large clumps, but fluid still cloudy.

1-1,000: at 3 $\frac{1}{2}$ hrs., large clumps; tube is nearly clear.

1-10,000: negative at 3 $\frac{1}{2}$ hrs.; at 5 hrs., small clumps. At 5 hrs. none of the tubes are, however, perfectly clear.

TABLE V.

THE PRECIPITATING ACTION OF THE ANTI-B. NO. 1 SERUM UPON ITS CORRESPONDING FILTRATE AND UPON THE FILTRATES OF *B. TYPHOSUS*, *B. COLI*, AND OF VARIOUS OTHER SPECIES OF THE COLON-TYPHOID GROUP.

B. No. 1 filtrate: 0.5 c.c. 0.5	Anti-B. No. 1 serum: 0.10 c.c. 0.25	Instant cloudiness. Instant cloudiness; precipitum at room temperature forms in less than $\frac{1}{2}$ hr.
B. typh. filtrate: 0.5 c.c. 0.5	0.10 0.25	Instant cloudiness; at 1 $\frac{1}{2}$ hrs., fine precipitum. Instant cloudiness; at 1 $\frac{1}{2}$ hrs., fine precipitum; at 6 hrs., flocculent precipitum.
B. colon filtrate: 0.5 c.c. 0.5	0.10 0.25	After several hours, cloudiness. At 1 $\frac{1}{2}$ hrs., cloudiness; at 6 hrs., a finely flocculent precipitate.
B. psittacosis: 0.5 c.c. 0.5	0.10 0.25	Immediate cloudiness; reaction in both tubes complete at 6 hrs.
B. typhi murium: 0.5 c.c. 0.5	0.10 0.25	As above.
B. enteritidis: 0.5 c.c. 0.5	0.25 0.10	Cloudiness at 1 $\frac{1}{2}$ hrs.; precipitate at 6 hrs. Cloudiness at 1 $\frac{1}{2}$ hrs.; none at 6 hrs., but slight precipitate at 18 hrs.
B. icteroides: 0.5 c.c. 0.5	0.25 0.25	Both tubes show slight precipitate at 19 hrs.
B. dysenteriae— Flexner and Kruse: 0.5 c.c. 0.5	0.25 0.1	No cloudiness at 6 hrs. At 19 hrs., slight flocculi in both tubes.
Staphylococcus filtrate:		No reaction after 72 hrs.

In the hanging drop, the following reactions were obtained with the typhoid bacillus:

1-20; complete clumping at 1 hr.; also at 24 hrs.

1-200; complete reaction; clumps are small, but slightly motile. No free bacilli.

1-20,000; at $\frac{3}{4}$ hr. small motile clumps; free motile bacilli.

These agglutinative relationships are of considerable interest, especially in connection with those we have demonstrated to exist in the case of the precipitins.*

The onset of reaction and amount of precipitum developed in the filtrates of *B. psittacosis*,† and typhi murium and the homologous *B. No. 1* filtrate were similar, the typhoid filtrate perhaps giving a less copious precipitum. With *B. enteritidis* the reaction was less marked, although the addition of 0.25 c.c. of serum to 0.5 c.c. of filtrate gave rise to a copious precipitum at six hours. The filtrates of the dysentery bacilli gave a flocculent precipitate at nineteen hours with 0.1 c.c. and 0.25 c.c. of serum. It has thus been demonstrated with anti-*B. No. 1* that a serum adapted to one species of the colon-typhoid group precipitates, in dilutions of 1-6, the filtrates of many different species of this group.

*Castellani and Durham, Theobald Smith, H. Bruns and H. Kayser,¹⁵ and many others, have recorded numerous examples of agglutinative affinities between the members of the colon-typhoid group. Carl Sternberg has shown that antityphoid serum may agglutinate, in dilutions of 1-5,000, several examples of so-called paratyphoid bacilli obtained from potable waters. The interreactions described above between the typhoid bacillus and the anti-*B. No. 1* serum do not always occur, as shown later on. No antityphoid rabbit serum has been obtained which agglutinated the *B. No. 1*, except in 1-10 dilutions, although such sera frequently gave rise to more copious precipitates in the *B. No. 1* filtrates than did some of the weaker anti-*B. No. 1* sera, especially when tested in dilutions of 1-10 or 1-5. This statement does not apply to the reactions obtained with higher dilutions of serum, the homologous serum always precipitating its corresponding filtrate in higher dilution than does the heterologous serum.

†The *B. psittacosis* and *B. typhi murium*, both obtained from Král, cannot be differentiated from our *B. No. 1* by cultural tests. In fact, the *B. psittacosis* is agglutinated and its filtrate precipitated by the anti-*B. No. 1* serum quite as effectively as the homologous bacillus, in some tests even more so. The *B. typhi murium*, however, does not agglutinate in such high dilutions or furnish quite as copious precipitates as does the *B. No. 1*. Nevertheless, as detailed later on, this was determined only by a careful estimate of the maximal serum dilutions which determine precipitation. Whether an animal immunized against *B. No. 1* is also protected against these two bacilli of the intermediate group was not determined, although it seems most probable. The *B. enteritidis* is not agglutinated by the anti-*B. No. 1* serum at all; by the antityphoid sera often in dilutions of 1-100 and 1-200. (Durham has made similar observations upon the action of antityphoid serum on *B. enteritidis*.)

ANTICOLI SERUM.

Rabbit VI, between March 1 and April 2, 1902, received seven agar cultures: bled April 7.

TABLE V.

THE PRECIPITATING ACTION OF THE ANTICOLI SERUM UPON ITS CORRESPONDING FILTRATE AND UPON THE FILTRATES OF *B. NO. 1* AND *B. TYPHOSUS*, AND OF VARIOUS OTHER SPECIES OF THE COLON-TYPHOID GROUP.

Colon fil.: 0.5 c.c.	Anticoli ser.: 0.1 c.c.	Immediate cloudiness; at 1 hr., flocculi.	At 5½ hrs., flocculi still floating.	At 19 hrs., complete.
0.5	0.25	Immediate cloudiness; at 1 hr., flocculi.	Reaction complete.	
<i>B. typhi</i> murium fil.: 0.5 c.c.	0.10	At 1 hr., negative.	A 5½ hrs., cloudiness?	At 19 hrs., moderate flocculent precipitum in both tubes.
0.5	0.25			
<i>B. No. 1</i> fil.: 0.5 c.c.	0.10	Cloudiness?	Slight cloudiness in 0.25 c.c.	In 0.1 c.c., flocculent precipitum less abundant than homologous tube.
0.5	0.25			
<i>B. psittacosis</i> fil.: 0.5 c.c.	0.10	Cloudiness?	Marked cloudiness.	In 0.1 c.c., cloudiness; 0.25 c.c., marked cloudiness.
0.5	0.25			
<i>B. typhoid</i> fil.: 0.5 c.c.	0.10	Cloudiness?	Marked cloudiness.	Both tubes show moderate amount of precipitum.
0.5	0.25			
<i>B. enteritidis</i> fil.: 0.5 c.c.	0.10	Negative.	Slight cloudiness in both.	Both tubes show moderate amount of precipitum.
0.5	0.25			
<i>B. icteroides</i> fil.: 0.5 c.c.	0.10	At 1 hr., negative.	At 5½ hrs., negative.	At 19 hrs., negative.
0.5	0.25			
<i>B. dysenteriae</i> fil., Kruse and Flexner: 0.5 c.c.	0.10	At 1 hr., negative.	At 5½ hrs., negative.	Very slight reaction in both tubes.
0.5	0.25			
<i>Staphylococcus</i> fil.: 0.5 c.c.	0.10	At 1 hr., negative.	At 5½ hrs., negative.	In 0.25 c.c. tube, a slight precipitate on sides.
0.5	0.25			

The anticoli serum thus causes precipitation, not only in the filtrates of bacilli belonging to the intermediate group, but also in the filtrates of the dysentery bacillus.

The anticoli serum thus precipitated the filtrates of various and distinct species of the intermediate and dysentery groups, except that of *B. icteroides* in dilutions of 1-5. Upon the staphylococcus filtrate it has no action.

SUMMARY OF SERIES II.

The results of the tests of Series II may be summarized briefly. The sera of three rabbits, immunized respectively to *B. typhosus*, the intermediate *B. No. 1*, and *B. coli*, precipitated in dilutions of 1-3 and also 1-6, not only their homologous, but also a large number of the filtrates of heterologous, species belonging to the same so-called colon-typhoid group. Precipitative relationships between the species of the same group are thus indicated.

SERIES III.

In order to confirm the observations made with the antisera of the rabbits of Series I and II, and also to enable us to widen the scope of our investigations, a third set of rabbits was adapted to the species of the colon-typhoid group, namely, *B. No. 1*, *B. typhosus*, and *B. coli*.

ANTI-B. NO. 1 SERUM.

Rabbit VII received six agar cultures, between April 4 to May 26, 1902; bled June 1. Agglutinating value: 1-5,000 to 1-10,000. No agglutinating action upon the typhoid or colon bacillus.

TABLE VI.

THE PRECIPITATING ACTION OF THE ANTI-B. NO. 1 SERUM UPON ITS CORRESPONDING FILTRATE AND UPON THE FILTRATES OF *B. TYPHOSUS* AND *B. COLI*.

B. No. 1 filtrate: 0.5 c.c. 0.5	Anti-B. No. 1 serum: 0.25 c.c. 0.10	At $\frac{1}{2}$ hr., slight cloudiness; at 4 hrs., fine precipitation; at 10 hrs., copious precipitation in both tubes, fluid being clear and sterile.
B. typh. filtrate: 0.5 c.c. 0.5	0.25 0.10	At $\frac{3}{4}$ hrs., slight cloudiness; at 4 hrs., not as marked as in homologous filtrate; at 10 hrs., the amount of precipitum is similar to above set.
B. coli filtrate 0.5 c.c. 0.5	0.25 0.10	No reaction at 4 hrs.; very slight reaction in both tubes at 24 hrs.; at 48 hrs., the tube with the larger amount of serum contains a copious precipitum.

ANTI-B. TYPHOID SERUM.

Rabbit VIII received, from April 10 to June 12, 1902, nine twenty-four-hour old agar cultures; bled June 17. Agglutinating value between 1-5,000 and 1-10,000. No agglutinating reaction upon *B. No. 1* (1-10, complete in four hours; 1-100, negative).

TABLE VII.

THE PRECIPITATING ACTION OF THE ANTITYPHOID SERUM UPON ITS CORRESPONDING FILTRATE AND UPON THE FILTRATES OF B. NO. 1 AND B. COLI.

Typhoid filtrate: 0.5 c.c. 0.5	Antityph. serum: 0.25 c.c. 0.10	Cloudiness in several minutes in both tubes; at 1 hr., small flocculi; at 4 hrs., copious precipitum, fluid clear; at 29 hrs., amount of precipitum similar in both tubes.
B. No. 1 filtrate: 0.5 c.c. 0.5	0.25 0.10	At 1 hr., opalescence and cloudiness; at 4 hrs., precipitation not quite as marked as with the homologous filtrate; at 29 hrs., the two sets of tubes are similar.
B. coli filtrate: 0.5 c.c. 0.5	0.25 0.10	Negative at 4 hrs.; at 19 hrs., large flocculi in the 0.25 c.c. serum tube. In the 0.10 c.c. tube scanty precipitum at 48 hrs. The reaction was slower and much less copious than with the other filtrates.

The tests with fresh anticoli serum could not be made owing to the death of the rabbits during the course of adaptation.

The tests made with the antisera of rabbits of Series III confirm the previous observations of Series I and II in regard to the precipitating action of the serum of a rabbit adapted to one species, upon the filtrates of two other species of the colon-typhoid group.

RECORD OF FURTHER TESTS MADE WITH THE ANTISERA OF SERIES II AND III, WITH THE SAME AND WITH ADDITIONAL FILTRATES.

The precipitating action of the antisera of the rabbits of Series II and III adapted to the three species of the colon group upon filtrates of other species of this same group which have not as yet been referred to, will now be given, for brevity, in tabulated form, the sera of Series II having been kept two months; that of Series III, several weeks.

SUMMARY.

Table VIII indicates that a highly active antiserum, "anti-B. No. 1," induces precipitation in a large number of heterologous filtrates (even after preservation for two months in cold storage). On the other hand, an antiserum, "antityphoid," originally much less active than the above anti-B. No. 1 serum, as indicated by its low agglutination value, 1-1,000, possesses only a slight precipitating action upon its corresponding and none upon heterologous filtrates.

TABLE VIII.

THE PRECIPITATING ACTION OF ANTI-B. NO. 1 AND ANTI-B. TYPHOSUS SERA UPON THEIR CORRESPONDING FILTRATES AND UPON THE FILTRATES OF HETEROLOGOUS SPECIES OF THE COLON-TYPHOID GROUP.

(Test made June 18, 1902.)

Filtrate	Anti-B. No. 1 Serum. Rabbit V, Series II. Agglutinating Value, 1-20,000. Bled April 8, 1902	Antityphoid Serum. Rabbit IV, Series II. Agglutinating Value, 1-1,000. Bled, April 2, 1902
0.5 c.c. B. coli: 1 3-4 10 1902. B. typhosus: 1 3-4 10 1902.	0.25 c.c. Negative at 48 hrs. Cloudiness at 4 hrs.; slight precipitum at 18 hrs.; more marked at 48 hrs.	0.25 c.c. Negative at 48 hrs. Negative at 18 hrs.; slight precipitum at 48 hrs.
B. No. 1: 1 3-4 10 1902.	Cloudiness at 1 hr.; slight precipitum at 4 hrs.; abundant precipitum at 18 hrs.	Slight precipitum at 48 hrs.
B. Schottmüller:* Stamm Seemann: 4 11-6 6 1902. Stamm Müller: 4 11-6 6 1902.	Cloudiness at 1 hr.; well-marked precipitum at 4 hrs. Cloudiness at 4 hrs.; slight precipitum at 48 hrs.	Negative at 48 hrs. Negative at 48 hrs.
B. Gwyn: 4 11-6 4 1902.	Cloudiness at 1 hr.; well-marked precipitum at 4 hrs.	Negative at 48 hrs.
B. Cushing: 4 11-6 4 1902.	Opalescence at 4 hrs.; distinct precipitum at 18 hrs.	Negative at 48 hrs.

TABLE IX.

THE PRECIPITATING ACTION OF ANTI-B. NO. 1, ANTI-B. TYPHOSUS, AND ANTI-B. COLI SERA UPON THEIR CORRESPONDING FILTRATES AND UPON THE FILTRATES OF HETEROLOGOUS SPECIES OF THE COLON-TYPHOID GROUP.

(Test made June 21, 1902.)

Filtrate	Anti-B. No. 1 Serum. Rabbit VIII, Series III. Agglutinating Value, 1-5-10,000. Bled June 1, 1902	Antityphoid Serum. Rabbit VII, Series III. Agglutinating Value, 1-5-10,000. Bled June 17, 1902	Anticoli Serum. Rabbit VI, Series II. No Agglutinating Value. Bled April 7, 1902
0.5 c.c. B. Schottmüller: Seemann.	0.1 c.c. At ½ hr., cloudiness; at 19 hrs., copious precipitum.	0.1 c.c. At ½ hr., cloudiness; at 19 hrs., copious precipitum.	0.1 c.c. Slight precipitum at 48 hrs.
Müller.	Negative at 4 hrs.; slight cloudiness and precipitum at 19 hrs.	Negative at 4 hrs.; slight precipitum at 19 hrs.	Negative at 4 hrs.; slight precipitum at 19 hrs.
B. Gwyn.	Slight precipitum at 19 hrs.	Slight precipitum at 19 hrs.	Negative at 48 hrs.
B. Cushing.	Cloudiness at ½ hr.; marked precipitum at 4 hrs.; copious precipitum at 18 hrs.	Cloudiness at ½ hr.; marked precipitum at 4 hrs.; copious precipitum at 18 hrs.	Small amount of precipitum at 24 hrs.
B. psittacosis.	Cloudiness at ½ hr.; copious precipitum at 4 hrs.; most marked reaction of all.	Cloudiness at ½ hr.; copious precipitum at 4 hrs.	Same as above.
B. typhi murium.	Same as above.	Same as above.	Same as above.
B. icteroides.	Negative at 4 hrs.; small amount of precipitum at 19 hrs.	Negative at 4 hrs.; at 19 hrs., small amount of precipitum.	Negative at 48 hrs.
B. enteritidis.	Cloudiness at ½ hr.; moderate amount of precipitum at 4 hrs.	Moderate amount of precipitum at 19 hrs.	Slight amount of precipitum at 24 hrs.

*The filtrates of Schottmüller, Gwyn, and Cushing were obtained from meat-infusion broth. The writer is personally indebted to Dr. Schottmüller for his cultures.

The filtrates psittacosis, typhi murium, icteroides, and enteritidis, were alkaline to litmus paper, and were obtained from Liebig's meat-extract peptone-salt broth, grown for three months at 37° C. and then passed through Berkefeld filters.

SUMMARY.

Table IX demonstrates the fact again that highly active antisera adapted to one species precipitate the heterologous filtrates of various species belonging to the same group. The anticolon serum which had been kept for seventy-five days produced no reaction, or only slight precipitates, in the heterologous filtrates. It must be borne in mind that, even when fresh, this anticolon serum did not produce copious precipitates in its corresponding filtrate.

The Müller, Gwyn, and icteroides filtrates gave the scantiest reactions. The most copious precipitates were furnished by the Seemann, psittacosis, and typhi murium filtrates, which reacted with the antityphoid and anti-B. No. 1 sera in serum dilutions of 1-6 as copiously as did the antisera in their corresponding filtrates. The onset of reaction, cloudiness, is practically instantaneous in the corresponding filtrates, whereas in the heterologous filtrates it is delayed for fifteen to twenty minutes or more.

A STUDY OF THE PRECIPITATING VALUE OF SERA ADAPTED TO SPECIES OF THE COLON-TYPHOID GROUP FOR THEIR HOMOLOGOUS AND HETEROLOGOUS CULTURE-BROTH FILTRATES. A DETERMINATION OF THE MAXIMAL LIMITS OF PRECIPITATION OF ANTISERA FOR THEIR CORRESPONDING AND HETEROLOGOUS FILTRATES.

In order to determine the limits of the precipitating action of antisera for their corresponding and heterologous filtrates, the following series of tests were made, at first in serum dilutions of 1-50 (5 c.c. of filtrate and 0.1 c.c. of serum), November 8, 1902:

TABLE X.

THE PRECIPITIN REACTIONS OBTAINED WITH ANTI-B. NO. 1, AND ANTITYPHOID SERUM
* DILUTIONS OF 1-50, IN THEIR CORRESPONDING FILTRATES AND IN THE FILTRATES OF
HETEROLOGOUS SPECIES OF THE COLON-TYPHOID GROUP.

Filtrate	Anti-B. No. 1 Serum. Agglutinating Value, 1-10,000. Bled Oct. 24	Antityphoid Serum. Agglutinating Value, 1-20,000. Bled Oct. 24
B. typh. 5 c.c.	0.1 c.c. Negative up to 24 hrs.	0.1 c.c. At $\frac{1}{2}$ hr., slight cloudiness; at $2\frac{1}{2}$ hrs., much cloudiness; at 22 hrs., abundant precipitate.
B. No. 1.	Slight cloudiness at $\frac{1}{2}$ hr.; at $2\frac{1}{2}$ hrs., much cloudiness; at 22 hrs., heavy precipitate.	Negative up to 4 and 24 hrs.
B. coli.	Negative up to 22 hrs. and later.	Negative up to 22 hrs.; at 48 hrs., a faint cloudiness.
B. typh., Pfeiffer.	Negative at 4 and 24 hrs.	Identical reaction to that given by the typhoid filtrate above.
B. dysenteriae: "Flexner." "Kruse." "Seward."	Negative up to 4 and 24 hrs. Negative up to 4 and 24 hrs. Negative up to 4 and 24 hrs.	At 22 hrs., slight cloudiness. At 22 hrs., slight cloudiness. At 22 hrs., slight cloudiness. No precipitate develops.
B. Cushing.	Negative at $2\frac{1}{2}$ hrs.; at 22 hrs., moderate amount of precipitate.	Negative at $2\frac{1}{2}$ hrs., at 48 hrs., and later.
B. Gwyn.	Negative up to 22 hrs.; at 48 hrs., small amount of precipitate.	Negative up to 4 and 24 hrs.*
B. Schottmüller: "Seemann."	Negative at 2 hrs.; small amount precipitum at 22 hrs.; at 48 hrs., considerable precipitum.	Negative at 22 hrs. and later.
"Müller."	Negative at 22 hrs.; perhaps slight cloudiness and small amount of precipitate at 48 hrs.	Negative at 48 hrs.
B. psittacosis.	Slight cloudiness at $\frac{1}{2}$ hr., which has increased in amount at $2\frac{1}{2}$ hrs.; at 22 hrs., a copious precipitate.	At $2\frac{1}{2}$ hrs., slight cloudiness; at 22 hrs., cloudiness and small amount of precipitate, which at 48 hrs. has increased.
B. typhi murium.	At $2\frac{1}{2}$ hrs., very slight cloudiness; at 22 hrs., distinct and moderate amount of precipitate; more at 48 hrs.	Negative at 48 hrs. and later.
B. enteritidis.	Negative at 48 hrs. and later.	Slight cloudiness at 22 hrs.; no precipitate develops.
B. icteroides.	Negative at 22 hrs.; slight amount of precipitate at 48 hrs.	Negative at 22 hrs.; at 48 hrs. ?
B. suipestis.	Negative at 48 hrs. and later.	Negative at 48 hrs.

* Addition of 0.1 c.c. of both sera together gave copious precipitate at twenty-two hours.
All filtrates strongly alkaline to litmus paper.

SUMMARY.

The table indicates that the sera of rabbits adapted to one species of the colon-typhoid group precipitates in dilutions of 1-50, the filtrate of many, but not all, of the species in this group.

DETERMINATION OF THE MAXIMAL PRECIPITATION LIMITS OF THE ANTI-B.
No. 1 SERUM FOR ITS HOMOLOGOUS BACILLUS.

In order to determine the precipitation limits of the anti-B. No. 1 serum more accurately than was indicated by the above table, the following tests were made:

To each tube was added 0.9 c.c. of filtrate and 0.1 c.c. of serum, or its dilutions in sterile 0.85 per cent. saline solution.

TABLE XI.
ANTI-B. No. 1 SERUM; AGGLUTINATION LIMIT, 1-10,000.

B. No. 1 filtrate:	B. No. 1 serum:	
9 10 c.c.	1 20 c.c.	Cloudiness marked at 1 hr.
9 10	1 40	Faint cloudiness at 1 hr.
9 10	1 60	Faint cloudiness at 1 hr.
9 10	1 80	Faint cloudiness at 1 hr.
9 10	1 100	Negative at 1 hr.; negative at 48 hrs.
9 10	1 120	Negative at 1 hr.
9 10	1 150	Negative at 1 hr.
		At 20 hrs., 1 100 c.c., a very faint reaction;
		1 150 c.c., reaction is possibly present; at
		48 hrs., a distinct small flocculus had
		formed. Reaction thus occurred at 1-1,500.

The limit of the precipitating action of the anti-B. No. 1 serum for the homologous filtrate was thus shown to be 1-150. By a similar method the limits of the action of the serum upon the following filtrates of broth cultures of approximately similar lengths of incubation were found:*

B. psittacosis, 1-150.

B. typhi murium, 1-120.

B. Schottmüller (Seemann), and B. Cushing, 1-80.

B. Gwyn, 1-40.

B. enteritidis, 1-50.

The serum did not precipitate in a dilution of 1-50, the filtrates B. typhosus, B. coli, B. dysenteriae, "Flexner," "Kruse," and "New Haven" (non-mannite fractors) or B. icteroides. The B. Schottmüller (Müller) gave a questionable reaction with the above serum dilution.

DETERMINATION OF THE MAXIMAL LIMITS OF THE ANTI-B. TYPHOID SERUM
FOR ITS HOMOLOGOUS BACILLUS.

ANTITYPHOID SERUM.

Agglutination limit, 1-20,000.

The serum was tested as was the anti-B. No. 1 serum, as above described. The precipitation limit for the homologous typhoid filtrate was found to be 1-150; for the psittacosis, 1-140; the filtrates of three dysentery races and

*Two factors, it may here be stated, were always taken into account in determining the precipitating value of an antibacterial serum: the degree of adaptation of the animal to the bacterial species, and the age and abundance of the growth of the broth culture from which the filtrate has been obtained. In the series of tests which were made to determine the limits of the precipitating action of the antisera upon their corresponding and heterologous filtrates, the various filtrates were accordingly obtained from broth cultures of approximately the same age.

B. enteritidis, 1-50 (slight cloudiness), no reaction in the other filtrates being obtained with a serum dilution of 1-50.

THE PRECIPITIN REACTIONS OBTAINED WITH A FRESH ANTICOLI SERUM.
ANTICOLI SERUM.

Agglutination limit, 1-250.

Owing to unavoidable circumstances, no anticoli rabbit serum was at hand at the time the above tests were made. A rabbit which had received eight and a half agar cultures during a period of fifty-one days, however, furnished a serum of high precipitative powers. The serum agglutinated* the homologous colon bacillus completely in a dilution of 1-250 in seven hours. Another colon bacillus of the same type (non-saccharose splitter) agglutinated only in a dilution of 1-100. The *B. typhosus* "Coll." and the intermediates were not agglutinated in dilutions above 1-10 and 1-50, respectively.

DETERMINATION OF THE MAXIMAL PRECIPITATION LIMITS OF THE ANTICOLI
SERUM FOR ITS HOMOLOGOUS FILTRATE.

The following tests were made to determine the precipitating limit, that is, the maximal dilution, at which a reaction is obtained by the anticoli serum in its homologous filtrate:

Each tube contained 5 c.c. of filtrate, and to each was added 1/10 c.c. of the diluted serum.

TABLE XII.

Filtrate	Anticoli Serum	2 Hrs.	6 Hrs.	24 Hrs.	72 Hrs.
5 c.c.	1/25 c.c.	Negative.	Very slight.	Clear fluid with slight precipitate.	Fairly abundant precipitate.
5	1/50	Negative.	Negative.	Negative.	Clear, sterile fluid; slight precipitate.
5	1/100	Negative.	Negative.	Negative.	Clear fluid; slight, but typical precipitate.
5	1/250	Negative.	Negative.	Negative.	Negative.

The maximal dilution at which a reaction is obtained was thus found to be about 1-500.

THE PRECIPITIN REACTIONS OBTAINED WITH ANTICOLI SERUM DILUTIONS OF 1-50 IN ITS CORRESPONDING FILTRATE AND IN THE FILTRATES OF HETEROLOGOUS SPECIES OF THE COLON-TYPHOID GROUP.

The anticoli serum was next tested upon the following filtrates:

Each tube contained 5 c.c. of filtrate and 1/10 c.c. of the serum. Tests were made at 37° C.

*The low agglutinative value of the serum for the *B. coli* the writer does not believe can be ascribed to the recent isolation of the bacillus from the cadaver, for it had been cultivated for a year, about twelve generations; and, furthermore, a stock colon bacillus was likewise agglutinated by the serum only in low dilutions. It is a well-known fact that freshly isolated cultures which themselves are more or less inagglutinable do give rise to abundant production of immune bodies in animals. The inagglutinability of typhoid and pyocyaneus cultures of recent isolation has been described in EISENBERG's interesting article, to which the reader is referred.¹⁶

TABLE XIII.

Filtrate 5 c.c.	3 Hrs.	6 Hrs.	8½ Hrs.	12 Hrs.	24 Hrs.
B. Schottmüller: Stamm Müller	Negative.	Negative.	Negative.	Negative.	Negative.
Stamm Seemann	Negative.	Negative.	Negative.	Negative.	Negative.
B. enteritidis	Very slight cloudiness.	Slight.	Slight.	Slight cloudiness; no precipitate.
B. psittacosis	Negative.	Negative.	Negative.	Slight.	Considerable pre- cipitum; fluid clear.
B. icteroides	Very slight cloudiness.	Slight.	Slight.	Considerable pre- cipitum; fluid clear.
B. typhi murium	Negative.	Negative.	Negative.	Negative.	Slight precipitum
B. typhi	Negative.	Negative.	Slight.	Slight.	Slight precipitum
B. No. 1	Negative.	Negative.	Negative.	Negative.	Slight precipitum
B. dysenteriae: "Flexner"	Very slight cloudiness?	Very slight cloudiness.	Slight precipi- tum; fluid nearly clear.
"Kruse"	Very slight cloudiness.	Slight.	Cloudiness, quite marked	Slight precipi- tate; fluid clear.
"Flexner" (New Haven)	Very slight cloudiness.	Slight.	Slight.	Considerable pre- cipitate.
B. Gwyn	Negative.	Negative.	Negative.	Negative.
B. Cushing	Negative.	Negative.	Negative.	Considerable pre- cipitate; fluid clear.
B. proteus*	Negative.	Negative.	Negative.	Negative.
Sp. Metchnikovi	Negative.	Negative.	Negative.	Negative.
Sp. cholerae	Negative.	Negative.	Negative.	Negative; pre- cipitate?
Staphylococcus pyogenes aureus	Negative.	Negative.	Negative.	Negative.

B. coli (homologous) clouding within fifteen minutes; at six hours, slight precipitation and marked clouding.

The filtrates of the three dysentery races have nearly as copious precipita at twenty-four hours as the colon. At seventy-two hours precipita have increased in amount, in *B. No. 1*, Seemann, typhoid, icteroides, psittacosis (copious), typhi murium, enteritidis, and Müller (very slight); Cushing, quite copious; Gwyn, less so; staphylococcus, *Sp. Metchnikovi*, and *B. proteus*, filtrates absolutely negative.

The anticoli serum, we have already shown, gives a reaction in the colon filtrate in a dilution of 1-500. In a dilution of 1-50 the serum precipitates the filtrates of *B. enteritidis*, psittacosis, icteroides, typhi murium, *B. typhosus*, *B. No. 1*, *B. dysenteriae*, Flexner, Kruse, and New Haven (non-mannite fractors), and *B. Cushing*, after twenty-four hours at 37° C., *B. Gwyn* giving only a slight reaction. The filtrates of other bacterial groups, such as *B. proteus*, *B. prodigiosus*, the vibrios, cholerae and Metchnikovi, and the staphylococcus, are not acted upon.

* Referred to later on.

THE PRECIPITIN REACTIONS OBTAINED WITH ANTICOLI SERUM DILUTIONS OF 1-10 IN ITS CORRESPONDING FILTRATE AND IN THE FILTRATES OF HETEROLOGOUS SPECIES OF THE COLON-TYPHOID GROUP.

Each tube contains 1 c.c. of filtrate and 1/10 c.c. of anticoli serum.

TABLE XIV.

	15-20 Minutes	1½ Hour	18 Hours.	72 Hours.
Schottmüller: Seemann	Negative.	Cloudiness.	Like typhoid, but a little less.	
B. enteritidis	Cloudiness.	Marked cloudi- ness.	Same as above.	
B. typhi murium	Cloudiness.	Marked cloudi- ness.	Heavy precipitum, like B. No. 1.	
B. Gwyn	Cloudiness.	Cloudiness.	Less than enteritidis.	
B. Cushing	Cloudiness.	Cloudiness.	Less than enteritidis.	
B. psittacosis	Cloudiness.	Cloudiness.	About like B. No. 1.	
B. No. 1	Marked cloudi- ness.	Less than colon.	Like colon tube, only slightly less in bulk. Next to colon tube.	
B. typhi (collec- tion)	Marked cloudi- ness.	Less than colon.	Like B. No. 1, perhaps less.	
B. typhi Pfeiffer	Cloudiness.	Marked cloudi- ness.	Less than other typhoid fil- trate.	
B. coli	Most marked cloudiness of all.	Marked precipitum on sides, which settles out on agi- tating tube.	
B. icteroides	Negative.	Negative.	Very small amount; increased at 72 hrs.	
B. dysenteriae: "Flexner"	Slight cloudi- ness.	Marked cloudi- ness, but less than B. No. 1 and B. typh. (col.).	Flocculent precipita.	
"Krusc"	Slight cloudi- ness.	Flocculi.		
"New Haven"	Slight cloudi- ness.	Flocculi.		
Sp. Metchnik- kovi	Negative.	Negative.	Negative.	Negative.
Sp. cholerae	Negative.	Negative.	Negative.	Negative.
Streptococcus pyogenes	Negative.	Negative.	Negative.	Negative.
aureus.				
B. prodigiosus	Negative.	Negative.	Negative.	Negative.

With one to ten anticoli serum dilutions, after eighteen hours, little difference in the amount of precipitum formed is made out between the colon and typhoid, B. No. 1, B. enteritidis, B. typhi murium, and B. psittacosis filtrates. The onset of the reaction with colon filtrate is, however, immediate, whereas in the typhoid and B. No. 1 filtrates the cloudiness reaction appears after fifteen minutes. At seventy-two hours all tubes containing filtrates of the colon-typhoid group contain a considerable amount of precipitum. The icteroides filtrate contains the least; but this is accounted for by the fact that the growth obtained in the broth culture which furnished the filtrate was considerably less than

those of the other bacilli, with the possible exception of the *B. dysenteriae* "Flexner" filtrate, in which the amount of precipitum was also small.*

SUMMARY OR TABULATION OF THE MAXIMAL LIMITS OF PRECIPITATION
OBTAINED WITH THE THREE FRESH ANTISERA.

The maximal limits of precipitation of the three antisera in the various filtrates, so far as determined, are represented in the following table:

TABLE XV.

		Limit of Pre- cipitation
B. No. 1 filtrate.....	October 24: Anti-B. No. 1 serum — aggluti- nation limit, 1-10,000	1-150 1-150 1-120-150
B. psittacosis filtrate.....	"	1- 80
B. typhi murium filtrate.....	"	1- 80-100
B. Schottmüller, "Seemann" filtrate.....	"	1- 40
B. Cushing filtrate.....	"	1- 50
B. Gwyn filtrate.....	"	
B. enteritidis filtrate.....	"	
B. typh. filtrate.....	October 24: Antityphoid serum — aggluti- nation limit, 1-20,000	1-150 1-140
B. psittacosis filtrate.....	"	
B. dysenteriae, Flexner, Kruse and New Haven filtrate.....	"	1- 50
B. colon filtrate.....	November 25: Anti-colon serum — agglutina- tion limit, 1-250	1-500 1- 50 1- 50 1- 50 1- 50 1- 50 1- 50 1- 50 1- 50 1- 50 1- 50
B. enteritidis filtrate.....	"	
B. psittacosis filtrate.....	"	
B. icteroides filtrate.....	"	
B. typhi murium filtrate.....	"	
B. typhoid filtrate.....	"	
B. No. 1 filtrate.....	"	
B. Cushing filtrate.....	"	
B. dysenteriae, three races, filtrate.....	"	

IV. THE PRECIPITATING ACTION OF THE SERA OF RABBITS
ADAPTED TO VARIOUS RACES OF THE *B. DYSENTERIAE*
SHIGA, AND ALSO TO OTHER SPECIES OF THE SO-CALLED
DYSENTERY GROUP, WHICH FERMENT MANNITE, IN THEIR
RESPECTIVE CULTURE FILTRATES.

The tests made with antidysenteric rabbit sera are now to be described, for although the *B. dysenteriae* doubtless belongs to the colon-typhoid group, it presents many points of contrast, as regards its fermentative and pathogenic characters, to the species

* The results obtained with this anticoli serum are in marked contrast to those recorded by KRAUS in the *Wiener klinische Wochenschrift* (1901, 14, p. 693) which emphasize the specificity of the precipitating action of the serum of animals adapted to various races of the colon bacillus.

heretofore mentioned; namely, *B. typhosus*, *B. coli*, and the dextrose splitters of the intermediate group. The three races of *B. dysenteriae* employed for the production of antisera and filtrates were a *B. dysenteriae* (known as Flexner), the *B. dysenteriae*, Kruse, and the *B. dysenteriae*, New Haven (Duval). Mannite is not fermented by these races, which accordingly belong to the Shiga type of the *B. dysenteriae*. Unfortunately, only a few experiments could be made with those species or races of bacilli which have been mistaken for the *B. dysenteriae* Shiga; namely, those species which ferment mannite and monosaccharids, and those which, unlike the former race, ferment in addition maltose, dextrin, and saccharose. Both these races, unlike the *B. dysenteriae* Shiga, have only slight pathogenicity for rabbits.*

Difficulty was experienced in immunizing rabbits to the three races of the *B. dysenteriae* Shiga in our possession, living cultures being pathogenic, whereas boiled cultures in our hands conferred only slight protection, and the serum of rabbits adapted to boiled cultures yielded only traces of agglutinating and precipitating substances. A precipitating serum was, however, obtained finally in a rabbit with repeated doses of living cultures of *B. dysenteriae* "New Haven."

ANTIDYSENTERY RABBIT SERUM.

Rabbit received about ten agar cultures of *B. dysenteriae* "New Haven," between June 4 and August 12, 1902. Serum collected August 19.

Agglutinating value: 1-1,000.

1-10: distinct agglutination at $\frac{3}{4}$ hr.; complete at 4 hrs.

1-100: distinct agglutination at $\frac{3}{4}$ hr.; at $1\frac{1}{2}$ hrs., agglutination farther advanced than the dilution tube 1-10; complete at 4 hrs.

1-500: at $1\frac{1}{4}$ hrs., indistinct clumps; at 4 hrs., settling, heavy deposit at bottom; complete at 8 hrs.

1-1,000: slight settling at $1\frac{1}{4}$ hrs.; at 4 hrs., like 1-500; complete at 8 hrs.

No reaction in higher dilutions at 8 hours.

In the hanging drop with a dilution of 1-100, small clumps are formed at seven hours. With a dilution of 1-200, small clumps developed at one-half hour, which did not increase in size. The serum agglutinated the *B. dysenteriae* "Kruse" in a dilution of 1-500.

The filtrate of the *B. dysenteriae* "New Haven" was obtained from a broth culture of beef extract, grown for one hundred and two days at 37° C.

*The reader is referred to the interesting article of HISS upon the differential fermentative peculiarities of the various species which have been thrown temporarily into the so-called dysentery group;¹⁷ also LENTZ.¹⁸

TABLE XVI.
THE PRECIPITATING ACTION OF THE ANTIDYSENTERIC SERUM UPON ITS HOMOLOGOUS FILTRATES.

Filtrate "New Haven"	Antidysen. Serum "New Haven"	
1 c.c.	1 c.c.	Immediate clouding, and precipitation in a few minutes; complete at 4 hrs.
1	1/4	Immediate reaction.
1	1/10	Cloudiness developed after 15 min., at 37° C.; at 4 hrs., small flocculi on sides of tube.
5	1/10	No reaction at 10 hrs.; at 22 hrs., a flocculent precipitum.

The serum thus agglutinated in a 1-1,000 dilution and precipitated in a dilution of 1-50, which represents practically its maximal limit of precipitation.*

TABLE XVII.
THE PRECIPITATING ACTION OF THE ANTIDYSENTERIC SERUM UPON ITS HETEROLOGOUS FILTRATES.

1 c.c. colon filtrate + 1	c.c. dysenteric serum:	Immediate cloudiness; precipitum at 1 1/4 hrs.
1 " " " + 1/4 "	" "	Immediate cloudiness; precipitum at 1 1/4 hrs.
1 " " " + 1/10 "	" "	No instant reaction; faint cloudiness at 1 1/4 hrs.; precipitum at 4 hrs.
5 " " " + 1/10 "	" "	Negative after 72 hrs.
1 " B. No. 1 " + 1/4 "	" "	Immediate cloudiness; at 4 hrs., abundant precipitum.
1 " " " + 1/10 "	" "	Immediate cloudiness; at 4 hrs., cloudiness and slight precipitum.
5 " " " + 1/10 "	" "	Negative at 72 hrs.

The typhoid filtrate reacted like the B. No. 1 filtrate. A prodigious filtrate was tested, with negative results.

SUMMARY.

The reactions above noted are not quantitatively as marked as that obtained with the homologous filtrate. The antidysenteric serum in a dilution of 1-50 failed to precipitate the filtrates of colon,

*Through the kindness of Professor Flexner, we were able to test the precipitating action of the serum of a horse which had undergone adaptation for four months with the dysentery bacillus—the culture "New Haven" isolated from a case of dysentery in an epidemic at New Haven.

Filtrate of B. dysenteriae "Flexner," 1/2 c.c. 1/10 and 1/4 c.c. horse serum.
Filtrate of B. dysenteriae "Kruse," 1/2 c.c. 1/10 and 1/4 c.c. horse serum.
Filtrate of B. dysenteriae "New Haven," 1/2 c.c. 1/10 and 1/4 c.c. horse serum.
Tubes kept at 37° C.

No reaction at six hours, but after twenty-four hours a small amount of flocculent precipitate had developed. The serum added in the same proportions did not precipitate the filtrates of any of the colon-typhoid group in our possession. The low agglutinating value, 1-200, by microscopic test, of the horse serum, reasonably accounts for the failure to obtain precipitation in any other than the dysenteric filtrates, especially as it has been noted that the typhoid, colon, and our intermediate B. No. 1 antisera precipitated dysenteric filtrates.

typhoid, and B. No. 1. Thus a reaction was obtained with a serum dilution of 1-10, but not in 1-50, in these heterologous filtrates.

The *B. dysenteriae* "New Haven" having been shown to possess precipitative relationships for the *B. typhosus*, *B. colon*, and the intermediate *B. No. 1*, in the tests to be described the precipitative relationships between several species or races of the dysentery group were studied.

THE AGGLUTINATING AND PRECIPITATING ACTION OF THE SERA OF RABBITS ADAPTED TO THREE SPECIES OF THE DYSENTERY GROUP UPON THEIR CORRESPONDING AND UPON HETEROLOGOUS FILTRATES.

The three species of the dysentery group are classified (Hiss) according to their fermentation reactions in the following manner: (1) The *B. Shiga* ferments dextrose and other monosaccharids; but not mannit, maltose, dextrin, or saccharose. (2) The *B. "Y"* (Hiss) ferments monosaccharids and mannit, but not maltose, dextrin or saccharose. (3) The *B. "Baltimore"* (same type as Flexner's "*Manila*" and "*Harris*") ferments monosaccharids, mannit, maltose, dextrin, and saccharose.

Two dysentery filtrates were employed—a Kruse filtrate (90 days at 37° C.) and a *B. "Y"* (Hiss) filtrate (184 days at 37° C.). Through the kindness of Dr. Hiss, three antisera were placed at the writer's disposal—a "*Shiga*," a *B. "Y"* (Hiss), and a "*Baltimore*" antiserum. The "*Shiga*" antiserum and the "*Kruse*" filtrate may be considered homologous, since the *B. Shiga* and the *B. Kruse* are non-mannit factors and have similar agglutinating limits. For the *Baltimore* antiserum no homologous filtrate was at hand.

The following agglutination tests (Table XVIII) were made by Prof. Hiss with the three antisera of rabbits and also with an anti-typhoid serum* (Macroscopic method, readings at twenty-four hours at 37° C.).

SUMMARY.

Agglutination relationships were thus found to exist between the mannit splitters, the *Baltimore* and "*Y*" cultures. Since the normal agglutinins of rabbit serum occasionally reach 1-100 for each of these species, an agglutination reaction below 1-100 has no significance.

* These agglutination results were kindly placed at the writer's disposal for the sake of completeness of the experiments, although they are to appear later in an article by Prof. Hiss on agglutination in the "dysentery" group.

TABLE XVIII.
AGGLUTINATION TESTS.
(Tests made June 23, 1903.)

Shiga serum, bled June 17:	Shiga, 1-1,600-3,200. Baltimore, 1-400. "Y," 100. } Only a little above nor- Typhoid, 100. } mal rabbit serum.
Baltimore serum, bled May 20:	Shiga, 1-100. Baltimore, 1-3,200. "Y," \pm 400. Typhoid, \pm 100.
"Y" serum, bled April 30:	Shiga, -100. Baltimore, +1,600. "Y," 6,400. Typhoid, +100.
Typhoid serum, bled April and May:	Shiga, -100. Baltimore, +100-200. "Y," +100. Typhoid, +6,000.

PRECIPITATION TESTS.

The following precipitin reactions were made with the four antisera and the three filtrates:

TABLE XIX.

Fil. Kruse, 5.0 c.c.	0.1 c.c. "Y" serum.	Cloudy at 6 hrs.; same at 24 hrs.
" " 5.0 "	0.1 " Shiga "	Cloudy at 6 hrs.; same at 24 hrs.
" " 5.0 "	0.1 " typh. "	Negative at 24 hrs. and 48 hrs.
" " 5.0 "	0.1 " Balt. "	Negative at 24 hrs. and 48 hrs.
" " 0.9 "	0.1 " "Y" "	Cloudy at 1 hr.; small precipitum at 24 hrs.
" " 0.9 "	0.1 " Shiga "	Cloudy at 40 min.; small precipitum at 24 hrs.
" " 0.9 "	0.1 " typh. "	Negative at 24 hrs. and later.
" " 0.9 "	0.1 " Balt. "	Negative at 24 hrs. and later.
Fil. "Y," 5.0 c.c.	0.1 " "Y" "	Clouding in 40 min.; at 6 hrs., cloudy; slight precipitum at 24 hrs.
" " 5.0 "	0.1 " Shiga "	Clouding in 40 min.? at 6 hrs., cloudy; slight precipitum at 24 hrs.
" " 5.0 "	0.1 " typh. "	Negative at 24 hrs.
" " 5.0 "	0.1 " Balt. "	Negative at 24 hrs.
" " 0.9 "	0.1 " "Y" "	Almost immediate clouding, more at 40 min.; at 22 hrs., small precipitate.
" " 0.9 "	0.1 " Shiga "	Cloudiness at 40 min.; at 22 hrs., small precipitate.
" " 0.9 "	0.1 " typh. "	Negative at 24 hrs.
" " 0.9 "	0.1 " Balt. "	Negative at 24 hrs.
Fil. typh., Pfeiffer, 5.0 c.c.	0.1 " "Y" "	Negative at 24 hrs. and later.
" " " 5.0 "	0.1 " Shiga "	Negative at 24 hrs. and later.
" " " 5.0 "	0.1 " typh. "	At 6 hrs., cloudiness; at 24 hrs., small precipitate.
" " " 5.0 "	0.1 " Balt. "	Negative at 24 hrs.
" " " 0.9 "	0.1 " "Y" "	At 45 min., negative; at 6 hrs., negative; at 24 hrs., very small amount?
" " " 0.9 "	0.1 " Shiga "	At 45 min., negative; at 6 hrs., negative; at 24 hrs., small amount.
" " " 0.9 "	0.1 " typh. "	At 45 min., negative; at 6 hrs., small precipitate; at 24 hrs., small flocculent deposit.
" " " 0.9 "	0.1 " Balt. "	Negative at 45 min., at 6 hrs., and at 24 hrs.

The "Kruse" filtrate is precipitated by the homologous Shiga and "Y" serum, but not by the Baltimore or typhoid serum. (The typhoid serum was of low value.)

The "Y" filtrate is precipitated by "Y" and Shiga, but not by the Baltimore and typhoid antisera.

The typhoid filtrate is precipitated only by its corresponding serum in serum dilutions of 1-50; in 1-10 dilutions, also by Shiga and possibly by "Y" serum.

SUMMARY.

Marked precipitative relationships were thus shown to exist between the Shiga and "Y" bacilli; whereas no agglutinative relationships, or only very slight ones, could be demonstrated with the help of the corresponding antisera, which possessed, however, moderately low agglutinating values.

V. THE PRECIPITINS DEVELOPED IN RABBITS ADAPTED TO VARIOUS BACTERIAL SPECIES WHICH DO NOT BELONG TO THE COLON-TYPHOID GROUP.

This part of our theme was undertaken, first, to determine the absence or presence of adaptation precipitins for species of bacteria other than those of the colon-typhoid group in the sera of rabbits that had adapted themselves to these species; second, to determine the specificity of the precipitins thus formed for a species of a given group by testing the antisera upon the filtrates of species belonging to other groups as well as classes of bacteria. We thus endeavored to throw further light upon the extent of the biological relationships which exist among bacterial species of different groups and classes of bacteria, by means of the precipitin reaction, in a way similar to that which Nuttall¹⁹ has so successfully employed to demonstrate the biological relationships which exist between animals belonging to various orders, classes and groups.

We shall now consider the precipitins which were developed by adaptation to various species of bacteria not hitherto or only cursorily recorded in the literature, as well as the ineffectual attempts that were made to obtain precipitins for certain species of bacteria.

I. THE PRECIPITINS DEVELOPED BY ADAPTATION OF RABBITS
TO *B. PROTEUS*.

The following tests were made with the serum obtained from a rabbit which had received six inoculations during a period of four weeks, at first with boiled and then with suspensions of living agar cultures. The agglutinating value of the serum was 1-5,000, complete in two hours; 1-10,000, incomplete at six hours. The precipitating action of the serum was slower and weaker than in the case of the antiprodigiosus and antistaphylococcus sera referred to below. One-fourth c.c. and 1/10 c.c. of the antiproteus serum added to 1 c.c. of the corresponding filtrate gave no reaction in five hours. Later a copious precipitum was formed in the tube containing the larger amount of serum and at twenty-four hours in the 1/10 c.c. tube the precipitum was still settling. One drop of serum to 1 c.c. gave no reaction at forty-eight hours at 37° C.

The antiproteus serum added to each of the filtrates in our possession likewise gave rise to no reactions, with the exception of the filtrate of the *Sp. Metchnikovi*, a slight reaction in this case being obtained at the end of twenty-four hours. On the other hand, the proteus filtrate was not precipitated or made cloudy by the antityphoid, coli, and *B. No. 1* sera added in the proportion of 1/4 c.c. of serum to 1/2 c.c. of filtrate, or by antistaphylococcus or antipyocyaneus serum.

With another antiproteus serum, with an agglutinating value of 1-500, taken from a dying rabbit which had received two inoculations of proteus emulsion, a similar and confirmatory series of observations was made, the precipitating action of the serum, however, being slight.

We may therefore conclude that no precipitating relationships exist between the *B. proteus* and the bacilli of the colon-typhoid group and the pyogenic cocci, but that in the class of spirillaceae the *Sp. Metchnikovi* may give reactions.

II. THE PRECIPITINS DEVELOPED BY ADAPTATION OF RABBITS TO
B. PRODIGIOSUS.

The antiprodigiosus sera yielded copious precipitates, and gave rise to the most rapid reactions which we have obtained with antibacterial sera.

The first serum was obtained from a rabbit inoculated at intervals, between April 4 and May 13, 1902, six, twenty-four-hour agar cultures being introduced into the peritoneum. The rabbit developed "snuffles" * May 16, and was therefore bled the next day. The serum had an agglutinating value of 1-10,000, complete in less than three hours. At 1-30,000 and 1-50,000 incomplete reactions were obtained.

THE PRECIPITATING ACTION OF ANTIPRODIGIOSUS RABBIT SERUM (No. 1)
UPON ITS HOMOLOGOUS FILTRATE.

One half c.c. and 1/10 c.c. of serum added to 1/2 c.c. of filtrate gave rise to instant cloudiness, heavy flocculi being formed in one-half hour, the reaction being complete in one hour.

THE PRECIPITATING ACTION OF VARIOUS ANTIBACTERIAL RABBIT SERA UPON
THE PRODIGIOSUS FILTRATE, AND THE ACTION OF ANTIPRODIGIOSUS RAB-
BIT SERUM (No. 1) UPON FILTRATES OF VARIOUS HETEROLOGOUS BAC-
TERIAL SPECIES.

The prodigiosus filtrate did not react with the various anti-bacterial sera in our possession; namely, anticolon, antityphoid, anti-B. No 1, and antidysentery; and no reaction followed the addition of the antiprodigiosus serum to filtrates of various bacilli, vibrios, and cocci; or this led to such small and inconstant precipitates, even when added in equal volumes, that these can be safely disregarded.

A second antiprodigiosus rabbit received eight twenty-four-hour agar cultures in the peritoneum, between April 7 and June 7, 1902, the rabbit being bled four days later. The macroscopic agglutination tests were instantaneous up to 1-500: 1-1,000, complete in less than one hour; 1-5,000, complete in less than two hours; 1-15,000, complete in six hours.

THE PRECIPITATING ACTION OF ANTIPRODIGIOSUS SERUM (No. 2) UPON ITS
HOMOLOGOUS FILTRATE.

One-half c.c. prodigiosus filtrate plus one drop of serum produced cloudiness and precipitation in two minutes, and at twenty-four hours a copious precipitum had formed. The limit of actual precipitation was obtained with a serum dilution of 1-300, the precipitum formed being very scanty.

* Adaptation with most of the cultures was rendered a tedious task during the winter months on account of a prolonged epidemic of "snuffles" in our rabbits. To forestall criticism, it may be mentioned here that only a few observations obtained with the sera of afflicted rabbits are recorded, the fact being noted in the text. Such sera were of value, however, in substantiating the results obtained with the sera of the healthy rabbits. The afflicted rabbits did not yield as potent precipitating and agglutinating sera, but the same relative results were found as with the antisera of healthy animals.

THE PRECIPITATING ACTION OF ANTIPRODIGIOSUS RABBIT SERUM (No. 2) UPON
THE FILTRATES OF VARIOUS ANTIBACTERIAL SPECIES.

The second antiprodigiosus serum was tested against various heterologous filtrates, each tube containing $\frac{1}{2}$ c.c. of the filtrate and $\frac{1}{4}$ c.c. of serum. The reactions obtained were slow, the twenty-four hour results alone being given. With filtrates of *B. typhosus*, *B. typhi murium*, *B. No. 1*, *B. psittacosis*, and *Sp. Metchnikovi*, a moderate amount of precipitum was formed, typical flocculi adhering to the sides of the tubes. The dysentery filtrates gave a small amount of precipitate. The filtrates of bovine and human tubercle bacilli, *B. icteroides*, and *Sp. cholerae*, were negative. At twenty-four hours all the fluids were clear and sterile, as found by cultural and microscopic tests of the fluid and precipitate.

The precipitin of this antiprodigiosus serum thus showing a somewhat marked affinity for the precipitable substances of these bacillary filtrates, one might be led to suppose that a strong precipitating serum would combine with the precipitable substance of any filtrate, whether of coccus or of bacillus, thus leading to the conclusion that there is no specificity in the precipitin reaction. This was readily disproved by the following test. The antistaphylococcus serum to be described later acted rapidly upon, and yielded a copious precipitum in, its corresponding filtrates.

TABLE XX.

Antiprodigiosus serum (No 2) 0.5 c.c.	Staphylococcus fil- trate 0.5 c.c.	After 48 hrs., at 37° C., absolutely negative.
Antistaphylococcus se- rum 0.5 c.c.	Prodigious filtrate 0.5 c.c.	After 48 hrs., at 37° C., absolutely negative.

Here, although both sera were extremely powerful, there is no interaction. In the course of the reactions between the antiprodigiosus serum and the above filtrates, but little of the precipitating substances of the serum were fixed, since addition of the prodigious filtrate to the tubes, when freed of the first precipitum that had formed after forty-eight hours contact at 37° C., gave rise to an immediate reaction. The reaction, although not delayed, was, however, never as copious. No attempt was made to calculate the amount of the precipitating substance of the serum which was thus fixed. This was the only antiprodigiosus serum obtained which precipitated heterologous filtrates of a different class, with the single exception of that of the filtrate of *Sp. Metchnikovi*.

A less actively precipitating serum of a rabbit treated with a sterile and cell-free prodigious filtrate likewise precipitated the *Sp. Metchnikovi* filtrate, a perfectly characteristic precipitate being formed in seventeen hours. The serum had no action on any other of the above filtrates, possibly because of its slighter precipitating action.

Our first antiprodigious serum obtained from a rabbit adapted by inoculation with living cultures, although of greater precipitating value for the corresponding filtrate than the serum of the rabbit inoculated with the prodigious filtrate, nevertheless failed to precipitate the same filtrate of *Sp. Metchnikovi*. From this fact it seems not unlikely that the *B. prodigious* develops several distinct precipitable substances, depending upon its environment. Thus, the filtrate of old broth cultures that was inoculated may have contained a larger quantity of the precipitable substance which gives rise to the formation of the special or partial precipitin of the *Sp. Metchnikovi*, than did the emulsion of the living bacilli with which the other rabbits had been inoculated. On the other hand, it is conceivable that there exists a difference, both quantitative and qualitative, in the cell receptors of different rabbits which give rise to the various partial precipitin haptines.*

Precipitative relationships between species of one group of Bacteriaceae and those of different groups of the same family and those of different families were thus found to exist. Since several equally actively precipitating antiprodigious sera did not precipitate the filtrates of the colon-typhoid group, it seems reasonable to assume that the cells of different rabbits develop precipitins which may vary qualitatively.

* Ascoli and von Dungern have noted qualitative differences in the hemo-precipitins developed in the sera of animals of the same and of different species. Von Dungern²⁰ has obtained almost conclusive evidence that the precipitable substance of the blood plasma of the Major squinado is composed of at least several haptophors which have distinct affinities, and that corresponding to the composite nature of this substance several precipitins are developed. The precipitins are thus composed of a number of partial substances, which are distinct from each other and react only with the special precipitable substance which has given rise to them. Joos²¹ has described two distinct agglutinins in anti-typhoid serum which, according to him, owe their origin to the presence in the typhoid bacillus of two distinct agglutinable substances. Joos's results seem capable of a different interpretation, however, from that which he has placed upon them.

III. THE PRECIPITINS DEVELOPED BY ADAPTATION OF RABBITS TO THE SPIRILLUM CHOLERAE AND METCHNIKOWI.

In the class of Spirillaceae only two species were investigated.

The first series of rabbits treated with cultures of *Sp. cholerae asiaticae* and *Sp. Metchnikovi* were unsuccessful, the sera having little precipitating action. The cholera culture was an old stock culture which had become non-pathogenic to guinea pigs and to rabbits. The *Sp. Metchnikovi* was, however, still pathogenic. Rabbits were finally adapted which yielded precipitating sera.

The anticholera rabbit was inoculated with twelve agar cultures (twenty-four hours' growth) in the course of ten weeks. (Agglutination test was impossible on account of extensive pseudo-clumping in broth cultures and precipitation of the emulsions of cholera cultures.) The anti-Metchnikovi rabbit received nine agar cultures grown for twenty-four hours at 37° C., in the course of seven weeks. The serum possessed an agglutinating value of 1-500, complete in four and one-half hours. The cholera serum did not agglutinate the *Sp. Metchnikovi* above the limit of normal rabbit serum (1-10 being nearly complete in four and one-half hours; 1-100 negative at twenty-four hours).

The filtrates of both species were obtained from broth cultures grown at 37° C. for fifty-one days.

TABLE XXI.

THE PRECIPITATING ACTION OF THE ANTICHOLERA AND ANTI-METCHNIKOWI SERA UPON CHOLERA AND METCHNIKOWI AND TYPHOID FILTRATES.

0.5 c.c. Cholera fil.*	0.5 c.c. cholera serum	No reaction up to 6 hrs.; at 24 hrs., a small precipitum in all tubes.
0.5 " " "	0.25 " " "	
0.5 " " "	0.1 " " "	
0.5 c.c. Metch. fil.	0.5 c.c. cholera serum	No reaction at 6 hrs.; at 24 hrs., a slight reaction, perhaps less than above homologous series.
0.5 " " "	0.25 " " "	
0.5 " " "	0.1 " " "	
0.5 c.c. Metch. fil.	0.5 c.c. Metch. serum	Instantaneous clouding in all tubes; at 4 hrs., reaction complete in all tubes; at 24 hrs., copious precipitum.
0.5 " " "	0.25 " " "	
0.5 " " "	0.1 " " "	
0.5 c.c. Cholera fil.†	0.5 cc. Metch. serum	Instantaneous clouding in all tubes. In 0.5 c.c. and 0.25 c.c. tubes copious precipitum, which did not form in 0.1 c.c. tube until 6 hrs. In the two sets of tubes at 24 hrs., same amount of precipitum.
0.5 " " "	0.25 " " "	
0.5 " " "	0.1 " " "	
0.5 c.c. Typhoid fil.	0.5 c.c. Metch. serum	No trace of reaction after 48 hrs., at 37° C.
0.5 " " "	0.5 c.c. cholera "	

* In the set of tubes which contained cholera filtrate and serum, where a slight reaction was first obtained, the addition of 0.5 c.c. of Metchnikovi serum likewise produced immediate cloudiness and precipitation in a few minutes. At eighteen to twenty-four hours there was a copious precipitum, showing that the cholera serum had fastened no appreciable amount of precipitable substance in its own filtrate.

† A further addition of 0.5 c.c. of Metchnikovi serum to the mixtures of cholera filtrate and Metchnikovi serum produced immediate cloudiness and precipitation at one and one-half hours; at eighteen to twenty-four hours, copious precipitum; most in 0.1 c.c. tube. The precipita were slightly greater than those of the homologous series.

DETERMINATION OF THE MAXIMAL PRECIPITATION LIMITS OF THE ANTI-METCHNIKOWI SERUM FOR ITS HOMOLOGOUS AND FOR THE CHOLERA FILTRATE.

The maximal limit of reaction of the anti-Metchnikowi serum on its corresponding filtrate was then determined.

TABLE XXII.

5 c.c. filtrate	1 10 c.c. serum.	Cloudiness within several hours: precipitation occurs before 20 hrs.
5 c.c. filtrate	1 20 c.c. serum.	Cloudiness is formed within 10 hrs.; never reaches a distinct precipitation even at 72 hrs.
5 c.c. filtrate	1 40 c.c. serum.	Negative at 72 hrs.

Maximal dilution about 1-100.

With 5 c.c. of cholera filtrate and 1 10 c.c. of Metchnikowi serum, no reaction at seventy-two hours. Maximal dilution at which precipitation still occurred in the cholera filtrate was considerably under 1-50, the exact limit not being determined.

SUMMARY.

From these tests the following conclusions can be drawn: The serum of an animal adapted to a given species of spirillum may precipitate the filtrate of another species of spirillum, although it does not agglutinate the spirillum. Distinct precipitative relationships may thus be present unassociated with agglutinative relationships.

IV. THE PRECIPITINS DEVELOPED BY ADAPTATION OF RABBITS TO *B. DIPHTHERIAE*.

It has long been known that during the process of immunization to certain species of bacteria the animal organism does not respond, or only with difficulty, with the production of precipitins, although various other antibodies, such as antitoxins, may be developed. We shall briefly describe our ineffectual attempts to obtain precipitins for the *B. diphtheriae*, the *B. pyocyaneus*, and the human and bovine tubercle bacillus.

No attempt was made to immunize rabbits with diphtheria toxin, as Kruse and Nicolle could not determine the presence of precipitins in diphtheria antitoxin (although agglutinins have been described in horse antitoxin serum). Although abundantly established that precipitable substances are present in filtrates, "toxins," the amount of precipitins developed in the serum of animals treated with filtrates was found by the writer and by other observers to be insignificant in comparison with that obtained when living bacterial cells are introduced.

Rabbits were accordingly immunized with emulsions of agar cultures, grown for twenty-four hours, in the hope of obtaining a precipitating or anti-bacterial serum. A rabbit was finally immunized against many times the fatal dose of diphtheria culture, preliminary doses of antitoxic horse serum being given. (The diphtheria bacillus was kindly furnished by the Department of Health of the city of New York, and is the one commonly designated throughout the world as the American bacillus, or Bacillus "8.")

Agglutination value of the serum: An emulsion was made from a thirty-hour agar culture in sterile saline solution. Macroscopic test:

1-10: reaction distinct in 40 min.

1-100, 1-500, and 1-1,000: complete in 4 hrs. at 37° C.

1-5,000: showed a greater settling than did the 1-10,000 or the control tube.

A second test failed to confirm the result above obtained, the emulsion being unsatisfactory on account of the clumping and sedimentation in the control tube. A third trial on June 14, ten days after the first test, was completely negative even in a 1-10 dilution. It seems reasonable to concede that the serum had a relatively low agglutinating value, about 1-1,000. The failures recorded in the second and third tests may perhaps be attributable to the condition of the emulsion.

THE PRECIPITATING ACTION OF THE ANTIDIPHTHERIA RABBIT SERUM UPON THE DIPHTHERIA FILTRATE.

The addition of 1/4 c.c. and 1/10 c.c. of serum to 1/2 c.c. tubes of diphtheria antitoxin (acid reaction), grown for three months at 37° C., caused no precipitation after several days at 37° C. Negative results were likewise obtained with a filtrate of extracts in normal salt solution of fresh agar cultures, even when mixtures of equal amounts (1 c.c.) of the antiserum and the solution were used.*

RÉSUMÉ OF PREVIOUS STUDIES UPON THE PRECIPITINS DEVELOPED BY ADAPTATION OF ANIMALS TO B. DIPHTHERIAE.

Diphtheria precipitins have been developed by S. Wassermann²² in the serum of rabbits treated with 0.1 per cent. ethylin-diamin extracts of dried and pulverized diphtheria bacilli, previously killed by heat, 60° C., any toxic action being neutralized by administration of suitable quantities of antitoxin.

Wassermann thus claims to have obtained a precipitating antidiphtheria serum which, unlike antitoxin, contains a substance which reacts specifically upon the body substance (the precipitable substance) of the diphtheria bacillus. The advantage claimed by him for this method over that by which merely the untreated bodies of the bacilli are inoculated is that more of the precipitable substance can be introduced. The serum, he claims, furnishes a method of differentiating the pseudo- from the true diphtheria bacilli by agglutination and precipitation tests. Wassermann hopes that with such a "bactericidal" serum the diphtheria bacillus may be made to disappear from the throats of patients who have continued to harbor the bacilli during a prolonged convalescence. Wassermann's expectation is premature, unless

*The diphtheria bacilli of three potato tube agar slants were grown for three days at 37° C. and emulsified in 5 c.c. of sterile normal saline solution and filtered.

one concedes that the precipitin is an immune body on a par in its action with the lysins, or is always accompanied by such a body, on which the assumption of the bactericidal powers of the serum is based.*

Lipstein²³ has shown that by a procedure similar to the one employed by us—intraperitoneal inoculation of living diphtheria bacilli mixed with anti-toxin to neutralize the toxin—a strong agglutinating serum is developed in rabbits. The subject of the agglutinin in antidiphtheria serum, and the employment of such sera to differentiate the true diphtheria races from the pseudo-diphtheria races or species, cannot be entered upon, the reader being referred to the articles of Lipstein and Lubowski,²⁴ and to the admirable article of Josef Schwöner.²⁵

Schwöner casually mentions that he has observed specific precipitation of broth diphtheria filtrates after twenty-four hours at 37° C., with anti-diphtheria horse serum.

V. THE PRECIPITINS DEVELOPED BY ADAPTATION OF RABBITS TO *B. PYOCYANEUS*.

With our stock culture of the *B. pyocyaneus* we were unable to obtain precipitating substances in the serum of several immunized rabbits for its homologous filtrates. The bacillus employed corresponded in most of its characters to the classical description of the *B. pyocyaneus*. With cultures of other races of this bacillus precipitins have been developed by Eisenberg.²⁷

VI. THE PRECIPITINS DEVELOPED BY ADAPTATION OF RABBITS TO HUMAN AND BOVINE TUBERCLE BACILLI.

Our efforts to obtain precipitating substances in the serum of rabbits which had been infected with living or inoculated with dead cultures of human and bovine tubercle bacilli were likewise unsuccessful, so that our experiments may be briefly given.

Rabbits inoculated with cultures of human and bovine tubercle bacilli, and bled at various periods of time after inoculation or before death, failed to develop precipitins in their sera for tubercle filtrates.

Five per cent. glycerin broth cultures grown for several months at 37° C., in which the growth of bovine and human bacilli had been luxuriant, were used as the test fluids. The rabbits were inoculated with virulent cultures or with large amounts of the surface scum from tubercle cultures. The sera tested were obtained from rabbits in various stages of tuberculation, from

*This is an interesting commentary on the loose methods of expression frequently employed in connection with the immune bodies, especially since A. Lipstein²⁶ was not able to obtain any evidence of the formation of a suitable amboceptor in the sera of animals inoculated with living cultures, although the same sera possessed marked agglutinating properties.

a local abscess at the site of inoculation in the subcutaneous tissue, to those with an acute general miliary tuberculosis of the peritoneum with more or less involvement of the lungs, lymph nodes, kidneys, and other organs. No suggestion of a precipitating reaction was obtained by addition of the serum to their corresponding filtrates. Negative results were also obtained with Koch's extract of tubercle bacilli, which he employed to determine the agglutinating action of the serum of animals and patients afflicted with tuberculosis. The solutions were made with the preparation of *zerriebene Tuberkelbacillus* prepared by the Farbwerk vom Meister Lucius und Bruning, Höchst a. M., the directions given for Koch's *Agglutinations-Flüssigkeit* being closely followed. Our sera, added in various proportions, did not precipitate or cause cloudiness of this solution.

VII. THE PRECIPITINS DEVELOPED BY ADAPTATION OF RABBITS TO THE STAPHYLOCOCCUS PYOGENES AUREUS, TO THE PNEUMOCOCCUS, AND TO THE STREPTOCOCCUS.

In the class of cocci three species were investigated as to their capacity for developing precipitins in the serum of rabbits which had been adapted by inoculation of their cultures. The species were a *Staphylococcus pyogenes aureus*, a pneumococcus, and a streptococcus. The staphylococcus precipitin is first described. The pneumococcus and the streptococcus are described together for brevity, since by their inoculation no precipitating sera were formed.

a) THE PRECIPITINS DEVELOPED BY ADAPTATION OF RABBITS TO THE STAPHYLOCOCCUS PYOGENES AUREUS.

The staphylococcus used during the course of the experiments was highly pathogenic for rabbits, and it was only with care that these were finally rendered immune.* With the serum obtained from a highly immunized rabbit, the following experiments were made:

The rabbit received from March 10 to April 7 nine agar cultures into the peritoneum. The agglutination tests with the serum were made by the macroscopic method with emulsions of eighteen to twenty-four-hour agar cultures.

- 1-100 and 1-500: complete in 5 hrs.
- 1-1,000: nearly complete in 5 hrs.
- 1-5,000: complete in 18 hours.
- 1-10,000: negative after 24 hrs.

1-5,000 may be said to be the limit of the agglutination value of the serum.

* I am indebted to Dr. Wadsworth for the culture, which was obtained from a case of severe phlegmonous inflammation of the nose.

TABLE XIII.

THE PRECIPITATING ACTION OF THE ANTISTAPHYLOCOCCUS SERUM UPON THE STAPHYLOCOCCUS FILTRATE.

Staphylococcus filtrate:	Antistaphylococcus serum	Immediate cloudiness, well marked after 5 min.; in 1 hr., distinct precipitate in sets of tubes at room as well as at incubator temperatures; reaction complete at 5 hrs., the filtrate being clear.
1 2 c.c.	1 4 c.c.	
1 2 c.c.	1 10 c.c.	

DETERMINATION OF THE MAXIMAL PRECIPITATION LIMITS OF THE ANTISTAPHYLOCOCCUS SERUM UPON THE STAPHYLOCOCCUS FILTRATE.

The maximal limit of dilution of the antistaphylococcus serum at which precipitation occurs in the staphylococcus filtrate was found to be a serum dilution of 1-60. The serum likewise precipitated the filtered normal saline extracts of fresh agar cultures, as shown below.*

THE PRECIPITATING ACTION OF ANTISTAPHYLOCOCCUS SERA UPON VARIOUS HETEROLOGOUS FILTRATES.

The above serum and other antistaphylococcus sera were tested upon the filtrates already referred to of the colon-typhoid group, and also upon the filtrates of *B. proteus*, *B. prodigiosus*, *Sp. cholerae*, and *Sp. Metchnikovi*, with negative results.

b) THE PRECIPITINS DEVELOPED BY ADAPTATION OF RABBITS TO THE PNEUMOCOCCUS AND TO THE STREPTOCOCCUS.

The precipitins for the pneumococcus and streptococcus are evidently, as shown by the failure to obtain any evidence of their formation in immunized rabbits, only obtained, if at all, by special procedures. Our experiments may therefore be briefly summarized. Three rabbits were rendered highly immune to virulent cultures of the pneumococcus and of the streptococcus† grown upon agar. The antisera of the rabbits failed to produce cloudiness or precipitates in their corresponding filtrates when tested in the following proportions: 0.5 c.c. filtrate + 0.25 c.c. serum. It might be

* Emulsions of the three-day growth on three agar tubes in 5 c.c. of sterile normal saline solution, after standing for several hours at room temperature, were passed through Berkefeld filters. Equal parts of the filtered solutions and antiserum, when mixed, gave rise to no reaction after several hours; at eighteen hours (37° C.) a copious precipitum was present.

† I am indebted to Prof. Hiss for the streptococcus which was obtained from a severe case of cellulitis of the foot. The pneumococcus was virulent to rabbits, and was obtained from Dr. A. Wadsworth, to whom my thanks are also due. The filtrates were obtained from 5 per cent. glycerin meat-infusion broth, grown for two months at 37° C.

concluded from these tests that the pneumococcus and streptococcus do not possess precipitable substances, or that, if present, suitable receptors are not present normally, or are sessile, or are not formed during the process of adaptation of rabbits to these species of cocci.

The following tests with *Staphylococcus pyogenes aureus*, indicate, however, that precipitable substances are present in the streptococcus and pneumococcus, but that these substances do not diffuse readily from their cells into their glycerin-broth cultures. This assumption furnishes an explanation of the non-precipitation of the filtrates by their corresponding antisera, although the same antipneumococcus and antistreptococcus sera precipitated the staphylococcus filtrate:

THE PRECIPITATING ACTION OF THE ANTIPNEUMO- AND ANTISTREPTOCOCCUS SERA UPON THE STAPHYLOCOCCUS FILTRATE.

0.5 c.c. staphylococcus filtrate + 0.25 c.c. serum.

The tests carried out in the above proportions show the formation of a small quantity of whitish, somewhat granular precipitate after eighteen to twenty-four hours. The tests were repeated several times with confirmatory results, the precipitum obtained being found free from bacteria on cultivation and on examination of the coverslips. In confirmation of the specific character of the precipitum obtained may be cited the fact that neither the antipneumococcus nor antistreptococcus sera produced precipitates in any of the filtrates of the colon-typhoid group or the other filtrates in our possession.

If the slight reactions obtained above are granted to be those of precipitation—and there seems to be no reasonable ground for doubting the correctness of this supposition—it seems fair to suppose that the precipitable substances exist in the pneumococcus and streptococcus. These substances, however, do not diffuse into the filtrates, being retained in the bodies of the cocci. When the living cultures are inoculated, they are set free and induce the formation of precipitins in the sera of the rabbits. These precipitins, however, possess only a relatively slight affinity for the precipitable substance in the staphylococcus, and hence the small reaction. The antipneumococcus and streptococcus sera do not therefore, produce precipitates in their corresponding filtrates, since little or no precipitable substances are present, these sub-

stances being retained in the bacterial cell protoplasm and not diffusing throughout the ambient fluid of the broth.*

Furthermore, it is reasonable to assume that no, or only an insufficient amount of, precipitable substance was present in the filtrate upon which the precipitins of our antipneumococcus and streptococcus sera could act with the formation of a precipitum, for the strongly precipitating antistaphylococcus serum does not yield precipitates in the pneumococcus and streptococcus filtrates.

The growth in the broth from which these filtrates were obtained was not as abundant as that formed in the staphylococcus or in other bacillary filtrates. The filtrates of staphylococcus, on the other hand, are rich in precipitable substances, and the precipitins of the pneumo- and streptococcus antisera possessing a slight affinity for the staphylococcus precipitable substance, precipitation follows.

RÉSUMÉ OF PREVIOUS STUDIES UPON THE PRECIPITINS DEVELOPED BY ADAPTATION OF ANIMALS TO THE PNEUMOCOCCUS AND TO THE STREPTOCOCCUS.

Pneumococcus.—Neufeld²⁸ obtained evidence of the formation of precipitating substances in the pneumococcus antisera by means of special procedures. According to him, the precipitable substance resides exclusively in the bacterial cells—a statement apparently true for the pneumococcus. By means of the solvent action of normal bile on a fresh pneumococcus culture, which does not alter the specific nature of the constituents of the cocci, a drop sufficing usually to dissolve the cocci of several cubic centimeters of broth culture, a solution is obtained to which, if a serum which agglutinates pneumococci in a dilution of 1-15 is added in the proportion of 1-2, 1-4, 1-8, refracting hyaline masses, the size of red corpuscles, are seen to form in the hanging drop after a quarter of an hour. The masses increase in size and clump, forming later a precipitate visible to the naked eye. Normal rabbit serum does not bring about this reaction. According to Neufeld, a serum which agglutinates specifically a bacterial species will always cause a precipitation in a solution containing a sufficient amount of the bacterial constituents. He leaves it uncertain whether one substance induces both reactions, as Kraus first claimed, or whether two distinct substances which are formed simultaneously in the serum induce these phenomena. Neufeld adheres to Bordet's theory of agglutination. Under the influence of the agglutinin a peculiar coagulation process takes place on the surfaces of the bacterial cells, which in the case of the pneumococcus is accompanied by visible changes in shape, and, secondly,

*A somewhat similar explanation has been given by Neufeld for the pneumococcus, by Aronson for the streptococcus, and by Schwöner and Wassermann for the precipitable substances of the diphtheria bacillus which is extracted from the cell protoplasm with difficulty. In our explanation the supposition is that the separation is made by the animal organism.

by an alteration in the physical properties of bacteria which induce clumping and change of equilibrium.

Dr. A. Wadsworth,²⁹ in our laboratory, has confirmed Neufeld's observation concerning the precipitation by antipneumococcus serum of the solution of the pneumococci which have been dissolved by bile.

Streptococcus.—Aronson³⁰ has shown that antistreptococcus sera which do not precipitate the ordinary streptococcus broth cultures nevertheless precipitate extracts of the cocci in 1 per cent. ethylin-diamin solutions. With a powerful antistreptococcus horse serum (for details the reader is referred to the original) he obtained typical agglutination of streptococcus cultures. The agglutination test, made by the macroscopic method, was absolutely typical and caused only by a serum of high valency. Complete reactions were obtained with dilutions of 1-30; those of 1-40 were slightly cloudy, and the 1-50 dilutions were negative. The clumps formed in the effective dilutions, after shaking, soon settled again, unlike the sedimentation occurring in cultures which remain cloudy. A 1-40 reaction was obtained with Aronson's so-called twentieth normal serum. Aronson believes that the agglutinating power of serum is independent of its protective power.

Besides the agglutinins, the serum contained a specific precipitin, but special methods for its demonstration were necessary, as the serum did not precipitate streptococcus broth filtrates. The bacterial cell-bodies were dried and crushed in a little water, or better, with ethylin-diamin solution, as described by Aronson in a previous article.³¹ The extract was filtered through paper until clear. Addition of his immune serum, in proportion of 1-10, to the solution thus prepared, caused precipitation in several hours; whereas normal serum caused no cloudiness. The method resembles that described by Robert Koch³² for the demonstration of the agglutinating and specific substances in the serum of individuals and animals. Aronson justly remarks upon the impropriety of Koch's designating this method one for the demonstration of agglutination instead of precipitation. How a supposedly sterile and cell-free solution can agglutinate is certainly incomprehensible. Aronson believes that his process of extraction will reveal precipitins in the various antibacterial sera which have hitherto not been successfully demonstrated by merely testing the sera upon broth filtrates.*

VI. THE PRECIPITATING ACTION OF THE ANTI-B. NO. 1, ANTI-TYPHOID, ANTICOLI, AND ANTIDYSENTERY SERA UPON THEIR HOMOLOGOUS AND HETEROLOGOUS SALINE SOLUTION EXTRACTS OF YOUNG AGAR CULTURES OF SPECIES OF THE COLON-TYPHOID GROUP.

Pick claims to have extracted from the typhoid bacillus two distinct substances, which he designated by the term "bacterio-coagulin." The bacterio-coagulin A was obtained from old broth filtrates, and is precipitated by alcohol. The bacterio-coagulin K was extracted from the normal saline extracts of young agar

*Tchistovitch reports that Marmorek has observed the precipitation of streptococcus filtrates by antistreptococcus serum

cultures, and was not precipitated by alcohol from its aqueous solutions. Although Pick was not able to develop precipitins in the serum of rabbits which had undergone adaptation by the use of these substances when purified, his absorption tests led him to conclude that each of the two bacterio-coagulins nevertheless developed separate and distinct precipitins in the serum of animals adapted in the usual way to the typhoid bacillus. The action of these precipitins, sero-coagulins A and K, were specific, since they reacted only with their corresponding bacterio-coagulins. Granting that Pick's statements are correct, it would follow from our observations, referred to below, that precipitative affinities or relationships exist between the bacterio-coagulins A and K obtained from various species of the colon-typhoid group, the A body being found only in old broth filtrates, the K body only in the saline extracts of young agar cultures.*

Our observations may be briefly summarized. The sera previously referred to, which had been adapted to several species of the colon-typhoid group—namely, *B. typhosus*, *B. No. 1*, *B. coli*, and *B. dysentery*, "New Haven"—were employed. It was found that these antisera precipitated, not only the filtered normal saline solution extracts† of agar cultures, grown for three days at 37° C., of the homologous species, but also the extracts of heterologous species of this group. The reactions were slower in onset, and less copious precipitates were formed than in the case of old broth filtrates of these species.

VII. RELATIONSHIPS EXISTING BETWEEN THE BACTERIAL AGGLUTININS AND PRECIPITINS.

A résumé of the various arguments advanced concerning the identity or non-identity of the bacterial agglutinins and precipitins cannot be entered upon here. The following series of observations made with the antisera mentioned above and with other antisera may be briefly described, since they furnish further proof of the early views of French observers (Bordet, Tschistovitch) that

*Pick's statements regarding the specificity of bacterio-coagulins are in direct opposition to the results which the author has obtained in a series of experiments which will be referred to in a later article.

†The extracts were allowed to stand at room temperature or in cold storage, 5-8° C., for from 2-24 hours before filtration.

the bacterial agglutinins and precipitins are distinct substances. The antiserum of the B. No. 1, a dextrose fractor belonging to the intermediate "Durham" or hog cholera group, precipitated, it will be recalled, in high dilutions, not only the filtrates of the various species of this group, which has been subdivided into two types, corresponding to the two species of bacilli isolated by Schottmüller, and known as "Seemann" and "Müller" (Types A and B), but also in lower dilutions than the above, the following species, namely, the B. coli Escherich "non-saccharose fractor," B. typhi, and various species of the indefinite dysentery group.

The species of the intermediate group of the Seemann type, to which the B. No. 1 belongs—namely, B. "Seemann," B. psittacosis, B. Cushing, B. typhi murium, B. icteroides—possess marked agglutinative affinities, the species agglutinating with high dilutions of anti-B. No. 1 serum, the agglutinative and precipitative affinities exhibiting a striking parallelism. The species of the Müller type of the intermediate group—namely, B. Schottmüller, Müller, B. Gwyn, and B. enteritidis (type determination ?)—have slighter, and with some antisera practically no agglutinative affinities. The precipitative affinities of the species of this group, although less marked than those of the species of the "Seemann" type (1-10-40 instead of 1-80-150), nevertheless are relatively of a higher grade and are more general than are their corresponding agglutinative affinities, as mentioned above.

Our contention as to the distinctness of the agglutinins and precipitins is confirmed by our observations that several anti-B. No. 1 sera did not agglutinate B. typhi or the B. coli (Escherich type), although these sera precipitated copiously the typhoid and colan filtrates in dilutions above 1-10, but not in dilutions of 1-50.

In this connection the agglutinative and precipitative relationships of antisera developed for the two types of bacilli isolated by Schottmüller, for several species of the intermediate and dysentery groups are of interest. The anti-"Seemann" serum agglutinated B "Seemann" 1-100,000; B. paracolun [Libmann] and B. No. 1, 1-64,000; B. typhi murium, B. psittacosis, and B. icteroides, 1-32,000; B. Cushing and a hog cholera bacillus designated by Professor Smith* as "motile," 1-16,000; and the following species which belong possibly to the Müller type of the intermediate group: B. "Müller,"

* We are indebted to Professor Theobald Smith for his motile and non-motile species of hog cholera, both cultures being dextrose splitters belonging to the intermediate group.

1-50 (large motile clumps and scanty sedimentation, negative 1-200, microscopic method); *B. Gwyn*, 1-50, negative 1-200; and *B. enteriditis*, 1-200; also the following species: *B. suispestifer* (Král's) (hog cholera-Salmon-Smith), 1-16,000; *B. suissepticus* (Král's), 1-1,000 (?); and the non-motile species of hog cholera of Professor Smith, negative 1-50; *B. dysenteriae* "Y" Hiss, 1-250; *B. dysenteriae* Kruse, negative 1-50; *B. coli* (Escherich), 1-100; *B. typhi*, 1-100.*

Owing to unavoidable circumstances we were able to test the precipitating action of the anti-Seemann serum upon only two filtrates of the species mentioned above. The maximal limit of precipitation for the homologous *B. "Seemann"* filtrate was 1-100, for the *B. "Müller"* filtrate, 1-40.† We see again illustrated the independence of the agglutinating and precipitating activities of antisera, the "Seemann" serum precipitating the "Müller" filtrate in dilutions of 1-40, whereas it fails to agglutinate the *B. "Müller"* in dilutions of 1-50, the homologous bacillus "Seemann" being agglutinated in dilutions of 1-10,000.

With the anti-"Müller" serum the following observations were made: The serum agglutinated *B. "Müller"* 1-16-32,000, and *B. Gwyn* 1-8-16,000, but did not agglutinate in dilutions of 1-50, *B. "Seemann"*, *B. No. 1*, *B. psittacosis*, *B. icteroides*, *B. typhi murium*, *B. paracoloni* Libman, and *B. Cushing*. The motile species of Professor Smith were positive 1-50, negative 1-500; the non-motile species, negative 1-50; likewise *B. enteriditis*; *B. coli* "Escherich," positive 1-500; *B. typhi murium*, positive 1-500; *B. dysenteriae*, "Y" Hiss and *B. dysenteriae*, "Diamond" Hiss, both negative 1-50; *B. dysenteriae*, Kruse, positive 1-50.

On the other hand, the antiserum developed cloudiness and flocculi within ten hours in dilutions of 1-10 in the following filtrates of the species above mentioned, which it did not agglutinate in dilutions of 1-50: *B. Schottmüller* "Seemann," *B. Cushing* *B. No. 1*, *B. psittacosis*, and *B. dysenteriae* "Y" Hiss, as well as in its homologous filtrates.‡

We thus see that, although *B. No. 1* and *B. Schottmüller* "Seeman" cannot

*The tests were made in tubes of narrow caliber with agar cultures grown for 24 hours at 37° C., suspended in normal salt solution, 10 c.c. for one agar culture, readings being made at 2, 10 and 19 hours. Those recorded represent the extreme limits—microscopic clumps seen with low-power lens at 19 hours. Equal parts ½ c.c. of emulsion and serum dilution were employed.

†The filtrates were obtained from broth cultures of similar periods of incubation, 79 days at 37° C.

‡Although the writer is of the opinion that his contention as to the distinctness of the bacterial agglutinins and precipitins, and consequently of the corresponding substances (agglutinable and precipitable substances), has been placed on a sound basis, nevertheless he realizes that many points underlying the intimate processes of agglutination and precipitation are still unknown. To illustrate, the following agglutination limits of an anti-*B. No. 1* serum kept in cold storage for two years and greatly concentrated through evaporation, may be cited:

B. No. 1, 1-40,000, practically complete and clear at eighteen hours at 37° C.; microscopic clumps seen with aid of hand lens at 1-60,000. (No proagglutinoid zone at 1-20.)
B. psittacosis: complete, 1-20,000; microscopic, 1-80,000.
B. Schottmüller "Seemann": complete, 1-40,000; microscopic, 1-80,000.
B. Schottmüller "Müller": complete, 1-4,000; microscopic, 1-80,000.
B. Cushing: complete, 1-8,000; microscopic, 1-80,000.
B. Gwyn: complete, 1-2,000; microscopic, 1-80,000.

be differentiated by their agglutinative reactions or biochemical characters, they nevertheless develop antisera with marked agglutinating differences indicating differences in the precipitable substances of the two species of bacilli, which may be explained, possibly, by the multiplicity of their precipitable substances, or of their haptophor groups.

The following observations made upon the agglutinating properties of the anti-B. No. 1 serum for the typhoid bacillus are of interest.

An anti-B. No. 1 serum, with an agglutinating value of 1-10,000, agglutinated the typhoid bacillus in dilutions up to 1-2,000; another anti-B. No. 1 serum agglutinated the same typhoid bacillus incompletely in dilutions of 1-300; whereas a third antiserum failed to agglutinate in dilutions above 1-10. On the other hand, three different antityphoid sera possessed no agglutinating properties for the B. No. 1, although the three antisera possessed marked precipitating action upon the filtrates of the B. No. 1.*

Observations similar to those recorded above concerning the distinctness of the two substances as seen by the non-parallelism of their activities, expressed in terms of serum dilutions, were made in the case of the antityphoid sera which in dilutions of 1-10, and in greater dilutions in the case of some filtrates, precipitated all the filtrates of the species belonging to the colon-typhoid group, enumerated in the text, whereas the serum (1-20,000) agglutinated the B. enteritidis (limit, 1-300), the other species being clumped in much lower dilutions or in such as approach approximately the limits of normal rabbit sera.

To conclude: an anticolon serum (agglutinating value, 1-250) precipitated the colon-filtrate in a dilution of 1-500; and in a dilution of 1-50 all the filtrates of the colon-typhoid group mentioned above, except B. Gwyn and B. Schottmüller (Seemann).

*Many observers have called attention to numerous examples of the specificity of the agglutinins of various species of the intermediate group. Durham, Castellani, and other observers have, on the other hand, noted numerous examples of agglutinative relationships between the members of the colon-typhoid group. See BELJAEFF, "Ueber Paratyphus-Erkrankungen,"³³

The reader is referred to a most suggestive article upon the agglutination affinities of related bacteria by Theobald Smith and Reagh.³³ According to them, "there exist agglutination relationships between the pathogenic groups of bacilli which ferment dextrose. . . . This relationship is not brought out clearly unless the agglutinative limit of the various cultures is worked out with a serum agglutinating its specific bacillus in dilutions of one to one thousand and above."

On the other hand, the serum agglutinated only in low dilutions the homologous colon cultures, and was without action upon another culture of *B. coli*, as well as upon the intermediate *B. No. 1* and the *B. typhosus*.

A consideration of these observations leads us to the conclusion that the bacterial agglutinins and precipitins are distinct substances which owe their origin in the serum of adapted animals to different bacterial receptors or haptophor groups. According to Ehrlich's theory, the haptophor groups are alone responsible for the development of the immune bodies; hence the assumption that the substances in question are identical would leave wholly unexplained the fact that an antiserum may possess precipitative affinities of a high degree without or with only slight agglutinative affinities for a given heterologous species, or even for its homologous species, as in the case of one of the anticoli sera, or only in low dilutions with the other sera. On the other hand, if it be assumed that the substances owe their origin to different sets of receptors, we have only to assume that the haptophors (precipitable substances) which give rise to the partial precipitins* are more constantly present in the bacterial cell than are the corresponding haptophors (partial agglutinable substances) which give rise to the partial agglutinins.

VIII. CONCLUSIONS.

The following conclusions may be drawn from our researches:

1. The normal serum of the rabbit and of the ox, and various antibacterial rabbit sera, exert no precipitating action upon the usual peptone-salt meat infusions "or Liebig's extract," broth of slight alkaline or acid reaction.

Normal rabbit serum and ox serum do not precipitate bacterial broth filtrates. We are therefore warranted in drawing the conclusion that bacterial precipitins are absent in the serum of the rabbit and of the ox.

- 2 a) The sera of rabbits adapted respectively to four species of the colon-typhoid group, which species are distinguished from

* Ehrlich and Morgenroth have assumed the existence of partial hemolysins; Durham and Wassermann, of partial bacterial agglutinins; and von Dungern, of partial hemoprecipitins.

each other by definite and constant biochemical characters—namely, *B. typhosus*, *B. No. 1*, a member of the hog-cholera or intermediate group of Durham, *B. coli communis*, “Escherich,” and *B. dysenteriae* Shiga (New Haven “Duval”)—precipitate not only their homologous filtrates, but also the filtrates of the species above mentioned, as well as the filtrates of all the species of the colon-typhoid group which were tested by us; namely, the filtrates of *B. psittacosis*, *B. typhi murium*, *B. enteritidis*, *B. icteroides*, *B. paracolon* Gwyn, *B. paracolon* Cushing, *B. Schottmüller*, “Müller,” and “Seemann.”

The reactions obtained by the sera of rabbits adapted to one species of the colon-typhoid group in its homologous filtrate and in the heterologous filtrates vary in the time of onset of the reaction, as denoted by the appearance of cloudiness, and in the copiousness of the precipitum which finally develops, cloudiness developing more quickly, and the amount of precipitum formed being greater, in the homologous than in the heterologous filtrates. These differences are most strikingly exhibited when actively precipitating antisera are tested in higher dilutions; for an antiserum invariably precipitates its homologous filtrate in higher dilutions than the filtrates of heterologous species—a fact which may be made use of in the differentiation of bacterial species.

The precipitins for species of this group, cannot, however, be considered specific in the strict sense, but rather generic; for, as mentioned above, a serum adapted to one species precipitates in low serum dilutions the filtrates of heterologous species. The bacterial precipitins may thus serve to indicate genus or group relationship. The term “specificity” has merely a quantitative and not a qualitative value, and should be applied only in this broad manner, for the bacterial precipitins.

The quantitative differences in the precipitating action of the sera of rabbits which have been adapted to various species of the colon-typhoid group for the filtrates of homologous and heterologous species of this group are best explained, we believe, at least for the present, by the assumption that mutual bacterial receptors—precipitable substances—exist among the species of

one group of bacteria, which receptors give rise to the formation of partial precipitins in the sera of adapted rabbits.

b) The sera of rabbits adapted respectively to two species of the group of *Spirillaceae*—namely, *Sp. cholerae asiae*, and *Sp. Metchnikovi*—precipitate not only the homologous filtrate, but also the filtrates of the other species. The statements made above concerning the precipitins of the colon-typhoid group would seem to apply to the precipitins developed by the use of species of the genus of *Spirillaceae*.

c) In regard to the *Coccaceae*, we have reason to believe that precipitative relationships also exist between certain groups of this family, for slight precipitates were developed in the filtrates of *Staphylococcus pyogenes aureus* by antipneumococcus and antistreptococcus sera.

3. Precipitative relationships between species belonging to one group of *Bacteriaceae* for those of different groups of the same family, or for those of different families, such as the *Coccaceae* and *Spirillaceae*, as a rule, were found not to exist. Exceptions to this general rule were, however, noted. Thus, the serum of a rabbit adapted to a species of one group (*B. prodigiosus*) may precipitate the filtrates of many different species belonging to other groups (colon-typhoid), and also those of species (*Sp. Metchnikovi*) of a different family (*Spirillaceae*). The precipitative relationships are, however, limited. Thus the same antiprodigiosus serum had no precipitative action upon filtrates of species (*B. proteus*) of other groups of the same family (*Bacteriaceae*), or of a different family (*Staphylococcus pyogenes aureus*).

These facts lead us to believe that the precipitin reaction may serve to detect biological relationships between various groups and families of bacteria analogous to those which Nuttall and others have shown to exist in the animal kingdom.

4. From a consideration of the agglutinative relationships among members of the colon-typhoid group described by various observers, and from our limited number of observations and of the precipitative relationships described in our paper, the following conclusion may be safely drawn: The precipitative relationship between various species of one group of bacteria is a much

more intimate and constant one than the agglutinative relationship. The correctness of this assumption is supported and emphasized by the fact that the reaction of precipitation, as applied to bacterial filtrates, occurs only with low serum dilutions—as a maximum 1-500, but usually much lower; whereas agglutination readily occurs with thousandth dilutions. Furthermore, although agglutination and precipitation are usually closely associated phenomena, they are independent. In other words, the agglutinins and the precipitins are distinct substances.

In conclusion, the writer desires to acknowledge his debt of gratitude to Professor T. Mitchell Prudden and to Professor Philip Hanson Hiss, for much help and many suggestions in the preparation of this article.

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THE LIFE-HISTORY OF *TRYPANOSOMA LEWISI* AND *TRYPANOSOMA BRUCEI*.*

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INTRODUCTION.

IN connection with the more special work of cultivating trypanosomes various observations bearing upon the life-history of *Tr. Lewisi* and *Tr. Brucei* have been made. These two forms have been widely studied, and their life-cycles in the vertebrate host appear to be well known. The transmission of *Tr. Lewisi* from rat to rat by means of insect parasites, and of *Tr. Brucei* from one animal to another by means of the Tsetse fly, has been established. What changes the protozoa undergo in these insect hosts is an interesting problem which would seem worthy of further investigation. The recent paper of Schaudinn²⁷ in which he classifies two intracellular hematozoa as *Trypanosomata*, as a result of having found trypanosome-like stages in the insect host, would indicate the existence of an intracellular stage for the generally recognized trypanosomes and to call for proof that this stage has not been overlooked. Such a paper from so eminent an authority is sufficient proof of the uncertainty which still obtains in regard to the complete life-history of these organisms.† Our observations do not touch upon this problem, but have been chiefly confined to the trypanosome in its mammalian host and in artificial culture. It is the purpose of this paper to bring together these observations and correlate them with the previous work upon this subject.

CLASSIFICATION.

Doflein⁴ classifies the blood parasite discovered by Lewis¹⁴ in the wild rat of India as follows:

Stem, Protozoa.

Sub-stem, Plasmodroma.

Class, Mastigophora.

Sub-class, Flagellata.

Family, Trypanosomidæ.

Genus, Trypanosoma.

Species, Trypanosoma (Herpetosoma) Lewisi.

The generic identity of the rat trypanosome with that of the frog, *Trypanosoma sanguinis*, Gruby,⁶ has been established by Laveran

† Since this paper was written Novy and Mac Neal have reached conclusions, based on experiments with pure cultures of bird trypanosomes, which do not uphold the views of Schaudinn. The results obtained will probably appear in the next number of this JOURNAL.

and Mesnil,¹¹ so that the specific name *Trypanosoma Lewisi* may be finally accepted for this organism.

TRYPANOSOMA LEWISI.

For the study of its life-history there are several excellent guides. The pioneer work of Rabinowitsch and Kempner²⁴ has been followed up by Wasielewski and Senn,⁵⁹ and by the researches of Laveran and Mesnil,¹² and others of less note.

According to Lewis, this trypanosome is found only in the blood of wild rats, *Mus decumanus* and *Mus rufescens*. In the blood of the former species it occurs in many parts of the world. In India, Lewis found 29 per cent. of the wild rats infected with it. Carter² at Bombay found 12 per cent., and Lingard,¹⁵ likewise in India, found over 30 per cent. of wild rats infected. Crookshank³ observed the trypanosome in 25 per cent. of London rats examined by him. R. Koch⁵ at Daressalam found 10 infected rats out of 24 examined (41.7 per cent.). Rabinowitsch and Kempner²⁴ reported 41.8 per cent. at Berlin. At Paris, Laveran and Mesnil¹² found it in 2 out of 43 rats examined (4.7 per cent.). Voges²⁸ in South America examined numerous rats with negative results. Rouget²⁶ at Constantine in Algeria failed to find trypanosomes in wild rats. They were found also by Dutton at Bathurst, Senegambia; by Calmette at Lille, and by Buard at Bordeaux. Francis⁵ examined 60 wild rats from different parts of Washington, D. C., without finding the parasite. They have been found at Philadelphia, Detroit, Lincoln, San Francisco, and Manila. Of 107 rats caught in Ann Arbor 5 were found infected (4.67 per cent.).¹⁸ These five rats were caught at different times in the same barn. Lately another focus of the infection has been found here in a certain cellar. It is interesting to note in this connection that Lewis in his original paper called attention to the focal character of the infection. Others seem not to have observed this fact. We may conclude from the foregoing that the distribution of *Tr. Lewisi* is as wide as that of its host.

It should be noted, however, that *Tr. Lewisi* is by no means the only trypanosome to be found in the blood of the wild rat. Thus in India it is said that the rats harbor the surra trypanosome.

This may or may not be true, for the only fact established thus far goes to show that the trypanosome present is pathogenic for other animals than the rat, in which respect it differs from *Tr. Lewisi*. The possibility remains that several species of trypanosomes may be present in the wild rat. No identification can be considered as complete without a full study of the morphological, structural, pathogenic, and above all the cultural characteristics.

Trypanosoma Lewisi does not multiply indefinitely in the rat, but passes through a short period of multiplication which is followed by a relatively long stage in which the organism persists, but without any increase in numbers; further multiplication taking place only in a new host. In a few instances, particularly in young animals, multiplication of the parasite continues until the death of the rat results. The fact that rats may die of this infection was first observed by Wasielewski and by Jürgens.⁷ In our first paper¹⁸ we have recorded several deaths from this cause. In general, however, the host survives the developmental stage, and the trypanosomes then persist in the blood without multiplying for months, and gradually diminish in number and disappear. In one white rat the parasites were still numerous nine months after inoculation. This stage corresponds to that met with in Texas fever and in human tick fever, where the protozoa may persist in the blood of the animal for months after its recovery.

Transmission to a new host can take place by the bites of fleas and lice. Rabinowitsch and Kempner²⁴ report one case of transmission by fleas. As stated in our first paper (*loc. cit.*), we have frequently observed trypanosomes in the stomach of lice fed upon infected white rats. In one instance several such lice were transferred to a fresh young rat (February 4, 1904). Positive infection resulted, numerous trypanosomes being found in the blood after fourteen days and persisting for five weeks. Microscopical examination of these lice, living or crushed, frequently reveals the parasite, but no stages of development as yet have been made out. It seems probable that the louse merely carries the protozoon from one animal to the other.

Experimental inoculation is carried out by injecting infected blood intraperitoneally or subcutaneously. Infection usually

results in full-grown white rats, and almost invariably in young rats. For old rats intraperitoneal injection of a large dose is often necessary to produce the desired result. The multiplication at the point of injection is of doubtful importance. It is certain that the trypanosomes quickly gain entrance to the blood stream, and that the real multiplication occurs there. They may be readily found in the blood four hours after intraperitoneal injection of a large dose, and such individuals resemble in every respect the trypanosomes injected. If dividing forms are present in the injected material, they continue to divide, and a rich blood infection is quickly produced. If, on the other hand, only adult trypanosomes are injected, the first dividing forms are found on the fourth day, and the cessation of division occurs correspondingly later.

In the adult stage (Plate III, Fig. 1) *Tr. Lewisi* is 27–28 μ long, whip included, and $1\frac{1}{2}$ –2 μ wide. Unlike the dividing forms, the whole body takes part in the undulating movement. By the Romanowsky method or its various modifications, this stage stains much less readily than do the division forms. On comparison, the protoplasm stains a much lighter color and the mottling with colorless areas is more apparent. The posterior tip in particular is often feebly stained. This is in marked contrast to the readiness with which the division forms take the dye. In well-stained preparations of the adult stage the parasites are all of the same type. The length is very constant.

The smaller chromatin body, which has been designated by several names, such as basal body, micronucleus, centrosome, and blepharoplast, is situated about 3 or 4 μ from the sharp posterior tip. It measures 1 μ in length by $\frac{2}{3}$ μ in width, and is placed transversely, extending across the entire width of the cell at this point. The flagellum at times appears to originate in this body, but the study of more favorable specimens clearly shows an achromatic space between them, as has been pointed out by Laveran and Mesnil. That there is an intimate and firm connection, however, cannot be doubted, for these structures often remain attached together when all the rest of the cell has disintegrated. The flagellum extends in a rather smooth curve, along the convex

border of the parasite, supporting the undulating membrane, and is prolonged about 7μ beyond the anterior tip of the body as a free whip.

At approximately 11μ anterior to the blepharoplast is found the nucleus; this also is oval in form, measuring $2\frac{1}{2}\mu$ by $1\frac{1}{2}\mu$, with the long axis longitudinal. In structure it presents a chromatin network in a lighter nucleoplasm. At times a grouping of the chromatin into twelve chromosomes is apparent.

After injection into a new host, this adult form is found without any perceptible change for three days. Whether in every case it first enlarges and then divides, or begins division at once to form the small type of trypanosome, is not certain, as the large and small forms appear in the peripheral blood simultaneously on the fourth day. It seems probable, however, that enlargement precedes division. This is the case in culture. We may assume that the injected trypanosome first enlarges and tends, to some extent, to lodge in the internal blood capillaries, whence its smaller progeny can more readily escape. Aided by the constant high internal temperature, the development of such lodged forms would proceed a little more rapidly than in those freely circulating in the blood stream. An examination of streak preparations of the internal organs of rats at the end of the third day after inoculation often shows a larger proportion of developmental forms than does the blood, although the number of trypanosomes present is not greater than the blood content would account for. This has been noticed more particularly in the case of the adrenal. But, be that as it may, the fact remains that the large and the small forms appear in the peripheral blood of the tail at the same time.

In their pioneer work upon this subject, Rabinowitsch and Kempner²⁴ described three modes of multiplication: longitudinal division, transverse division, and segmentation. Wasielewski and Senn,²⁹ by study of living preparations and by employing an improved staining method, were able to show that the differences in arrangement of nuclei of the dividing cells, which was for the former workers a characteristic distinction between longitudinal and transverse division, were due to manipulation. Likewise they

showed conclusively that the characteristic feature of the segmentation, namely, "that division was initiated only after loss of the undulating membrane and flagellum and the rounding up of the parasite," did not exist in reality, and that Rabinowitsch and Kempner had been deceived by the stain employed. They thus established unequal longitudinal division as the only method of multiplication of *Tr. Lewisi*, and showed that apparent differences were nonessential variations of this one process.

Laveran and Mesnil¹² recognized two modes of multiplication: unequal longitudinal division (Group *a*), and rosette formation (Group *b*)—a simultaneous division into several coequal daughter-cells, no mother-cell being distinguishable. They were inclined to regard this latter as a variety of longitudinal division rather than as a segmentation.

Rabinowitsch and Kempner²⁵ in a later contribution accept Wasielewski and Senn's corrections for the most part, but still maintain that the rosette formation is a distinct mode of multiplication, and that the mother-cell is not to be recognized in such rosettes.

Our observations in regard to the division of *Tr. Lewisi* are similar to those of Wasielewski and Senn. The characteristic feature of the Group *b* of Laveran and Mesnil does not exist in reality. Unbroken multiplication rosettes show the mother-cell (Plate XIV, Fig. 2), and even when broken up, the division of the separate elements continuing, is still essentially unequal, as appears, in the same figure. It is only more difficult to distinguish between whips which have formed in rapid succession than between one of these and the old, full-grown whip. The essential inequality is always there, as must follow from the fact, as will be shown, that the whip multiplies, not by division, but by new formation.

Beginning then with the adult stage, we may say that the protoplasmic body increases in length, breadth, and thickness, and in staining power (Plate XIII, Fig. 3). The flagellum does not elongate, but becomes thicker and more readily stainable. The blepharoplast elongates slightly, the nucleus becomes larger and less definitely outlined, and its chromatin more loosely arranged,

so that often the nucleus is almost concealed by the densely staining protoplasm. The blepharoplast and the nucleus approach each other. In a living preparation this form is seen as a wide stiff spindle drawn along by the rapid vibratory motion of its relatively short flagellum. The motion is markedly different from that of the adult type. It may be worth noting that the whip, though relatively short, is by actual measurement of the same length as that of the adult trypanosome.

Division begins in the blepharoplast and flagellum, less frequently in the nucleus, and then only in the later stages of infection. It is frequently possible to make out the so-called division of the flagellum before that of the elongated blepharoplast is apparent. The split appears to extend along the whip to about its middle, at which point the daughter-whip breaks out to one side and is shorter than the other. The flagellum really does not split, but the shorter whip grows anew from a base near the blepharoplast (Plate XIII, Fig. 3). This fact is often apparent in blood stains, and has been proved in the case of *Tr. Brucei* by dissolving away the sheath of the flagella by alkaline sodium phosphate, in which case the short new whip is seen free with a pointed end, as in Plate XV, Fig. 4. In general the new flagellum is in relation with the more posterior of the two separating daughter-blepharoplasts. The nucleus divides directly.

The division of the protoplasm is assisted by the lashing of the free flagella. The two resulting cells remain attached longest at their posterior tips. Each daughter-cell proceeds to further division without waiting to enlarge, for an undetermined number of times, and the resulting cells are very small, 10–15 μ in length or even smaller. The larger one which retains the old whip usually proceeds to its second division more rapidly than the other.

The small cells which result from these repeated divisions (Plate XIV, Fig. 7) possess all the structure of the adult trypanosome. The blepharoplast and nucleus lie close together, but rapidly separate as the cell elongates. The undulating membrane is a rather rudimentary fold of the outer protoplasm, which elongates as the chromatin bodies separate. Various stages in the growth of these small forms are seen in Plate XIV, Fig. 3, which also

serves to illustrate the variety of size which *Tr. Lewisi* presents during its developmental stage in the rat.

In culture on blood agar *Tr. Lewisi* may give rise to much smaller forms. Cells with a body-length of 5 or 6 μ are common (Plate XIV, Fig. 8), and smaller forms, 2 or 3 μ in length, have been frequently seen. Such cultures after passage through a Berkefeld filter still infect rats.

In the first days of the developmental stage the daughter-cells separate as soon as formed, but later, about the sixth to the eighth day, they tend to remain attached at their posterior tips while continuing to divide, thus forming rosettes of four, eight, sixteen, or even more cells. The eight-cell rosettes are quite common (Plate XIV, Fig. 2). Their formation is due to the increasing stickiness of the trypanosome body and indicates a beginning antagonistic action on the part of the host. Finally, about the ninth or tenth day, dividing forms are no longer found, the trypanosomes assuming the slender form of the adult type.

At this time occurs a more or less marked agglutination of the trypanosomes. The posterior tips become very sticky and readily attach themselves to blood corpuscles or to each other. Two trypanosomes touching at their posterior tips stick and remain in contact in spite of the apparent effort to free themselves. Others catch by their posterior tips at or near this junction, and thus agglutinations of a hundred or more cells may be formed. In some rats all the trypanosomes are gathered into such agglutination rosettes at one time. In most cases, however, only a small number of the parasites are found agglutinated at any one examination. After a day or two the trypanosomes are again single and often as numerous as before, but gradually decrease till finally after several weeks they disappear entirely. In rare cases this agglutination results in the sudden disappearance of all the parasites. The phenomenon may be considered as a peculiar reaction of the trypanosomes to an immunity on the part of the host. It is less marked in the more susceptible young rats.

The contact of the two agglutinated trypanosomes is not merely at the extreme posterior tips, as described by Laveran and Mesnil,¹²

but most frequently the tips overlap each other somewhat (as in Plate XVI, Fig. 5), resembling the figures which Plimmer and Bradford²¹ have given as conjugations of *Tr. Brucei*. Changes in the smaller chromatin body, which they call the micronucleus, are difficult to make out and their interpretation is necessarily doubtful. At this stage and under high magnification, this body always presents a bilobed or hour-glass appearance. In stains of blood containing the agglutination rosettes many of the trypanosomes are separated in the act of spreading the film. In some instances fragmentation of the blepharoplast is distinct and certain, two or three chromatin bodies of unequal size being found at the posterior tip. We shall return to these problematical observations later under the subject of conjugation.

As stated above, the trypanosomes after agglutination separate again, as a rule, and continue single in the blood for some time. These adult forms are distinctly different from the dividing forms. The constant size, the feeble staining power of the protoplasm, and the compact and sharply outlined nucleus are in strong contrast with the features presented by the dividing forms and mark this as a distinct type of the organism. In the later stages of infection this adult type may be found, though rarely, undergoing a slow and apparently greatly retarded longitudinal division. We have observed this condition as late as the one hundred and tenth day of the infection. The trypanosomes in this case are larger than the others and contain two nuclei, one anterior to the other. They are quite different in appearance from the dividing cells of the developmental stage.

In the final destruction of these trypanosomes Laveran and Mesnil assign phagocytosis the chief rôle. By injecting trypanosomes into the peritoneal cavity of guinea pigs immunized to this organism, and subsequently examining the peritoneal fluid, they were able to find various stages of ingestion of the protozoa by the leucocytes. We have attempted to confirm this by a similar procedure. Instead of a phagocytosis, however, we could find evidence only of the immobilization and gradual solution of the trypanosomes in the peritoneal fluid. Stains made from time to time showed the protoplasm of the parasites paler than before,

until finally it seemed to have dissolved. The process appeared to be quite analogous to that met with in the well-known Pfeiffer's phenomenon. In other words, the trypanosomes disappear as the result of the presence and action of cytolytic agents rather than by phagocytosis.

CULTURE OF *TRYPANOSOMA LEWISI*.

The development of *Tr. Lewisi* may be followed a little more closely *in vitro* than in the living host, but the study is complicated by the presence of degenerating and dying forms. The difficulty in obtaining good stains offers another serious obstacle. The great advantage lies in the fact that the changes which take place in the living cell may be watched under the microscope. The Ranvier slide is the most satisfactory for this purpose, as it gives a thin layer between slide and cover, provides some air supply, prevents desiccation, and may be rendered free from contaminating organisms. Obviously very thin cover-glasses should be employed, so as to permit the use of the oil-immersion objective. The slide and cover-glass are first sterilized by flaming, then a ring of vaseline is applied around the outer edge of the circular well. A loopful or two of the fresh condensation fluid from a blood-agar tube is then placed in the center of the slide, after which a loopful of the culture is added, and the whole is covered with the sterile cover-glass. The study of such preparations was made in a warm room, kept at a constant temperature of 30° C. Under these conditions the trypanosomes in the preparations remain alive and can be kept under observation for two or three days.

A tube of the proper blood agar,¹⁸ inoculated with a drop of blood rich in adult forms of *Tr. Lewisi*, is capped and set aside at 30°. From time to time a loopful of the condensation fluid is removed for study. Within a few hours the trypanosomes are found agglutinated into small bunches of two, four, or more cells, all of which are very actively motile. No further change is apparent until the third day. Then dividing forms are found, large and small individuals and rosettes of four to one hundred cells.

In from three to four weeks the culture becomes quite rich, and various peculiar forms are met with, particularly round granular bodies of different sizes, whose formation may be watched under the microscope. They result from the contraction and rounding of an active long form, motion diminishing at the same time. Frequently two or more trypanosomes lying near each other coalesce to form one large sphere from which a corresponding number of whips extend out in various directions and lash feebly for some time. Repeated observation of such forms under the microscope has shown that they eventually lose all motion. These motionless round bodies might be considered as fertilized cysts, or something of that nature, from their method of formation, were it not for the negative experiments in cultivation and inoculation. Thus, repeated transplantation of different cultures rich in these forms, but free from motile trypanosomes, has always failed to produce another culture. Again, inoculation of animals with such material has yielded equally negative results. They must therefore be considered as dead and degenerated forms.

After growth for a year or so on artificial media the trypanosome seems to adapt itself to the new conditions. Frequently tubes are found in which veritable colonies can be seen upon the surface of the blood agar above the level of the condensation fluid. These are moist, raised, and glistening white. At first they were mistaken for bacterial colonies, but microscopic examination showed a mass of active trypanosomes in various stages of growth and degeneration.

CELL-DIVISION OF *TR. LEWISI* IN CULTURE.

A Ranvier slide preparation was made by mixing one loopful of an actively growing culture with two loops of fresh condensation fluid from a blood-agar tube, and studied in the warm room at a constant temperature of 30° C. A careful survey of the whole slide was first made by means of a mechanical stage, and the location of probable divisional forms noted. Division is best observed in the small rosettes of four cells. These have practically no progressive motion, and yet are simple enough for each cell to be clearly seen and followed throughout. After going over the whole slide a certain specimen was selected and kept under constant observation, hasty sketches being made from time to time as changes were observed.

In one experiment a rosette of five cells was selected (Plate I, Fig. 1). The cells in this case possessed little motion and were attached together loosely at their central tips. Sometimes a cell would appear entirely free for a time and later become attached again. All of the changes were slow enough to be easily followed, and there was no tendency in the group under observation to move any distance. A definite whip could be made out only on one cell, the largest. The others appeared to have no whips, or at most a short stub. They possessed a slow swaying motion. When observation was begun at 8:25 A. M., one cell (Fig. 1 *a*), was rounded and an indication of a division line was already present. A second cell, *c*, was also somewhat shortened. Five minutes later the division line in *a* was more certain and a transparent line had appeared in *c*. Five minutes later, at 8:35 A. M., the two parts of *a* had separated so as to leave no doubt, *c* was certainly dividing, and another cell, *d*, was growing thicker and shorter. The next five sketches were made at intervals of three minutes. At 8:41 A. M. (Fig. 5) the two daughter-cells of *a* were lengthening, *c* was completely divided, and *d* showed a faint division line. The fourth cell, *e*, was beginning to shorten and thicken. At 8:55 A. M. (Fig. 9) the three cells, *c*, *d*, and *e*, were all dividing, and the two daughter-cells of *a* had lengthened and become quite actively motile. The next sketch (Fig. 10), made fifteen minutes later, at 9:10 A. M., indicates the altered position due to their activity. At 9:39 A. M. (Fig. 12) the divisions were completed, and the rosette, now consisting of nine cells, was being actively twisted about by the increasing motion of its parts. The motion became so confusing that it was impossible to follow any one cell with certainty. Three hours later, however, it was possible to count at least twelve—sometimes there seemed to be fourteen—cells in the rosette. It appears, therefore, from this that in cultures the *Tr. Lewisi* may divide in an hour and proceed to a second division within four hours. Multiplication in the body must be more rapid than this, for here we were working with a temperature of only 30° C., not to mention other unfavorable conditions.

The position of the flagella in these rosettes was a puzzle for a long time. They are not to be seen on the peripheral ends of the cells except in rare cases, and then only in the very small rosettes. Rarely they can be seen directed centrally in the living preparation, and the peculiar motility of the cells, as if they were attached to each other by narrow waving stalks, suggests this position. However, the center of a rosette is not distinctly seen in the living preparation, and one cannot be certain that the whips are there. Opposed to this is the undoubted fact that the dividing rosettes in the living blood have their flagella on the periphery. To settle this point it was necessary to stain the whips, which was accomplished by the use of strong Romanowsky-Nocht mixtures.¹⁹ The resulting preparations are somewhat dirty in appearance, but they show conclusively that the whips are central in direction (Plate XIV, Fig. 4). The nucleus and blepharoplast are seen close together near the center of the cell. The flagellum is heavily stained and extends from near the smaller chromatin body toward the center of the rosette, where it is attached to the whips of the other cells.* We may consider such a mass to arise by successive divisions of a single cell, the new flagellum remaining attached to or entangled with the old, while the cell-body has completely divided. It is interesting to note in this connection that Schaudinn²⁷ has observed rosettes with centrally directed whips in the mosquitoes infected with the halteridium, *Hemoproteus noctuæ*. These he interpreted as agglutinations.

The cell-division is not exactly equal. One daughter-cell always projects slightly beyond the other during division, and when they have separated there is a difference in size—often very slight, however. This inequality is best seen in a single dividing cell which has become caught in some way. Such a cell which was found adhering to the cover-glass in a Ranvier preparation, with only its whip free and moving, was carefully watched. When first observed, the cell-body was oval in form and measured 9 by $4\frac{1}{2}$ μ . It shortened a little more, and in the

*In the recent publication of Laveran and Mesnil (*Trypanosomes et Trypanosomiases*, Paris, June, 1904, p. 77), which appeared since this paper was written, the authors describe and illustrate this rosette formation.

course of fifteen minutes a second short whip was observed at the base of the old one, and a division line was apparent in the anterior half of the body. Here on account of the fixed position of the object the inequality of the two daughter-cells was easily seen. Moreover, it is a fact that the difference in size is really more marked in the case of a dividing large free cell than is the case for the smaller cells composing a rosette. After another thirty minutes' observation the division was completed except for the attachment at the posterior tips.

Attempts to carry out similar observations upon dividing forms taken from the living host have failed. The change from living blood to blood-agar condensation fluid under a cover-glass put a sudden stop to all division. Cells caught in the very act of division made no visible progress, although the preparations remained alive for two days. Jürgens,⁷ however, appears to have demonstrated that there is an actual increase in numbers in cover-glass preparations.

TRYPANOSOMA BRUCEI.

The etiological factor in the Tsetse-fly disease or Nagana of Africa was discovered in 1895 by Bruce¹ in Zululand. He considered the parasite as either identical with or closely resembling that observed by Evans in India. Kanthack, Durham, and Blandford⁹ in their report upon this disease give a preliminary description of the protozoon. Plimmer and Bradford²⁰ in their preliminary note named the organism *Trypanosoma Brucei*. They outlined a very complicated life-history, including conjugation, formation of amœboid bodies, etc., besides multiplication by longitudinal and transverse fission. Laveran and Mesnil¹⁰ first clearly recognized the longitudinal division of the parasite and established it as the only method of multiplication in the mammalian host. They¹³ regard the conjugation described by Plimmer and Bradford as merely an agglutination phenomenon, and failed to find such agglutination in freshly drawn Nagana blood, but only after it had been some time out of the body. When the trypanosome blood was mixed with physiological salt solution faintly acidulated with acetic acid, they observed the rapid formation of agglutinations and their disintegration into granular

dead material. On the other hand, weak solution of soda prevented agglutination. Plimmer and Bradford²¹ in a later report confirm the division process as described by the French workers, abandon their transverse division, but still cling to their plasmodial and amœboid types. In this later paper they also describe and figure two special types of the organism—the large hyaline trypanosome, with feebly staining protoplasm and sharply outlined nucleus (Plate XVII, Fig. 2), and the very granular type with its protoplasm full of deeply staining granules (Plate XVII, Fig. 1). All these investigators used trypanosomes descended from those brought to England from Zululand in a living dog by Dr. Wag-horn in 1896.

Martini¹⁷ found *Tr. Brucei* in the blood of a Barba pony brought from Togo to the zoölogical garden at Berlin. Employing this material, he has confirmed the multiplication stages described by Laveran and Mesnil.

The virus which we have employed was obtained by Dr. Novy through the kindness of Dr. Thomas, of the Liverpool School of Tropical Medicine, and is the same as that used by the English and French workers. It has served for the cultivation experiments elsewhere described.¹⁹

In rats and mice this parasite produces death in from two to ten days, according to the amount of infective material injected. Development appears to proceed uninterruptedly, as a rule, from the time of inoculation until the blood is literally swarming with the protozoa, when death occurs. Sometimes there is an interruption to the progress of the disease. The rat, rarely the mouse, sometimes acquires a resistance, and the parasites actually decrease in number. This is only temporary, however; the vigorous multiplication soon returns and death invariably results. In these cases the disease is somewhat longer, lasting twelve to fifteen days.

In guinea pigs and rabbits the resistance is usually more marked. The parasites may become very numerous, and then diminish until the microscope no longer reveals their presence for long periods. They may thus increase and diminish in number several times. However, death always finally results. Our

guinea pigs have died in from ten to sixty days, rabbits in from twenty to forty days.

We have observed only one type of multiplication, unequal longitudinal division. There is a preliminary enlargement, so that the dividing cell is both longer and thicker than the average. The division process begins in the blepharoplast and flagellum, being apparent first in the latter, as a rule. The division of *Tr. Brucei* is essentially the same as that of *Tr. Lewisi*. The great apparent difference is due to three things. First, *Tr. Brucei* is continually dividing, growth and multiplication going hand in hand; as soon as a cell gets a little larger than the average it divides into two. In the rat trypanosome, on the other hand, the cell grows very large before division is initiated. Then successive divisions take place rapidly, growth fails to keep pace, and hence very small forms result. Second, the Nagana parasite does not shorten and lose its motion during division to any such extent as is seen in the rat parasite. Lastly, there is no sticking together of the daughter-cells to form multiplication rosettes of *Tr. Brucei*.

As to whether the flagellum really splits into two, for part or the whole of its length, or the new whip just grows out with its peripheral end closely applied to the old one, is hard to decide from the ordinary preparations. In stains of Nagana blood it appears actually to split, the forking of the old flagellum being very distinct. According to Laveran and Mesnil,¹⁰ the whip splits to the end of the undulating membrane and at times throughout its whole length. During the course of a series of experiments to test the effect of various salt solutions upon *Tr. Brucei* certain observations were made which throw an important light upon this question. One-fourth of a cubic centimeter of a molecular solution of the normal sodium phosphate was added to 0.75 c.c. of defibrinated rabbit's blood. To this mixture a drop of freshly drawn defibrinated rat blood rich in *Tr. Brucei* was added. After five minutes, microscopic examination showed many trypanosomes with the actively motile whips separated from the body except for their attachment near the blepharoplast. At the end of thirty minutes stained preparations were made, and it is in these that the true relations of the new whip formation seem to have been

found. The alkaline phosphate dissolves away the undulating membrane which sheathes the flagellum, and this is then set free. The early stages of division in which the flagellum appears to be branched near the blepharoplast, present in these preparations two entirely separate flagella, one of which is very short with a tapering tip (Plate XV, Fig. 4). It would seem, therefore, that the division of the old whip is apparent and not real, and that its sheath forms a path of least resistance for the new whip to grow out into. The new flagellum is formed entirely anew from the blepharoplast, and not by a division of the original whip, as has been heretofore supposed. This corresponds to the observations which have been made on the flagellum formation in the case of *Tr. Lewisi*.

As the new flagellum grows the blepharoplasts separate, the one in relation with it moving posterior to the other, as if the active whip growth pushed its base backward and its tip forward at the same time. The indefinitely outlined nucleus divides directly into anterior and posterior portions. The separation of the flagella from each other produces two undulating membranes which unite at the point of contact of the whips. As this point is pushed farther forward, the two undulating membranes come to occupy the opposite lateral borders of the cell, and a lighter line of thinner protoplasm appears running down the middle between them (Plate XV, Fig. 2). Such cells are often seen in the living preparation. The final division of the protoplasm takes place along this lighter line in such a way that the posterior tip of the mother-cell goes with the new whip and the greater part of the anterior protoplasm with the old whip. As the connecting band between the daughter-cells weakens they tend to slip on each other, the posterior one becoming more posterior, and in this way the cells separate from each other, the complete division being facilitated by the actively motile flagella.

Multiple divisions are not uncommon, three blepharoplasts, whips, and nuclei being present in one large cell at the same time. Simultaneous division into four is rare. As a rule, the daughter-cells separate and enlarge before dividing again, so that there is not the remarkable difference in size seen in the case of *Tr. Lewisi*.

When the protozoa are numerous in the blood three, more or less distinct, types can be recognized by the variation in the amount of granulation. The first or ordinary type presents a narrow body with a large elongated oval nucleus occupying the whole width of the body. The outline of the nucleus is indefinite. The protoplasm stains deeply and contains granules which stain like the nucleus or somewhat darker. These are found in the protoplasm anterior to the nucleus, few posterior to it.

Far less numerous are the larger faintly staining forms (Plate XVII, Fig. 2). In these the protoplasm is free from granules and stains a clear pale blue. The nuclei are compact, round, sharply outlined, and do not occupy the whole width of the cell. In staining character they bear a suggestive resemblance to the rat trypanosomes undergoing solution in the peritoneal fluid of the immunized guinea pig. If stained preparations are made from the blood of a Nagana mouse at intervals before and after death, the stages of disintegration of the protozoa may be studied. The nucleus of the trypanosome, dying under these conditions, becomes spherical; its protoplasm becomes progressively more feebly staining, and finally dissolves, leaving the round nucleus by the side of the flagellum. The blepharoplast remains attached to the latter. From this it would seem that the large hyaline forms of Plimmer and Bradford are really dying and dissolving in the living blood.

The third or very granular type is somewhat more rare, as a rule. Plimmer and Bradford at first considered them to be trypanosomes which had just completed the act of conjugation, but in their later paper they are inclined to question the correctness of this view. Thus far there is no very definite evidence in regard to their origin or meaning. It seems probable that some of them are degenerating forms as somewhat similar cells are found in old, dying cultures.

Although not so marked as in the case of *Tr. Lewisi*, yet agglutination of the Nagana trypanosome does occur. Collections of from two to five cells are readily found in the freshly drawn blood of the unusually resistant rats previously mentioned, this phenomenon being associated with a diminution in the number of

parasites present. A similar agglutination is also associated with the reduction in number of the parasites in guinea pigs.

Outside the body, agglutinations readily form, and this tendency is much increased by the presence of acids or acid salts. Laveran and Mesnil noted the marked effect of minute additions of acetic acid. Similar results are obtained with sodium hydrogen sulphate and sodium metaphosphate. Stained preparations of agglutinated material show peculiar changes in the attached posterior ends of the trypanosomes, especially interesting being the appearance of the blepharoplast. These will be discussed later under the head of conjugation.

CULTURE OF TR. BRUCEI.

The Nagana parasite may be grown *in vitro* by a procedure analogous to that used for the rat trypanosome.¹⁸ The results obtained have been published in two papers.¹⁹ A somewhat detailed discussion of the proper medium was given at the last meeting of the Michigan Academy of Science.¹⁶ In general, it may be said that the Nagana trypanosome is more exacting in its requirements, and that its cultivation is more difficult than in the case of the rat parasite. For isolation from the living animal a nutrient agar of the following composition is prepared :

Extractives of 125 g. chopped beef in distilled water	1000 c.c.
Agar - - - - -	20 g.
Pepton - - - - -	20 g.
Common salt - - - - -	5 g.
Normal solution Na_2CO_3 - - - - -	10 c.c.

The agar thus prepared is distributed into tubes or small Erlenmeyer flasks and sterilized in the autoclave. To one volume of the sterile melted agar, cooled to about 60° C., two volumes of naturally sterile defibrinated rabbit's blood are added, thoroughly mixed, and allowed to solidify. Tubes are allowed to solidify in the inclined position; flasks upright. Into the small amount of liquid which collects upon the surface of the blood agar, pure Nagana blood is inoculated, and here development takes place.

Examination in a few hours will show many small agglutinations of from two to six cells, many trypanosomes, however, remaining single. Careful daily microscopic examinations for the next fifteen days may show various forms and changes leading to the death of the trypanosomes. The formation of round bodies, the *mis en-boules* of Laveran and Mesnil, may be readily followed. The body of a trypanosome contracts into a spherical mass with its whip extending out and retaining a feeble motion for some time. Frequently two or more cells lying near each other are seen to coalesce into a large sphere with a corresponding number of whips. That these are death changes is indicated by the failure of such round bodies to infect animals or to give rise to another culture when transplanted to fresh medium. There

is a steady diminution in the number of protozoa until about the tenth to fifteenth day, when it is often an endless search to find one under the microscope. The disappearance may be final and the attempted culture a failure. In many cases they reappear between the twentieth and thirtieth day, and can then be successfully transplanted. In the most favorable first generations the trypanosomes can easily be found microscopically and an increase in number is then apparent as early as the tenth day. Transplantation from the first generation has been successful when made as early as the fourteenth day and as late as the fifty-second day. After an active multiplication of from four to fifteen days the number again diminishes, and the protozoa disappear finally at about the fiftieth day, often earlier.

For the succeeding cultures inoculated from this first generation, a blood agar with four times as much meat extractives is employed. Upon this the growth is more rapid and abundant, although subsequent degeneration and death is also hastened. The transplantation is performed by transferring a drop of the condensation fluid by means of a sterile Pasteur pipette. The cultures may be grown at room temperature, but a constant temperature of 25° C. is more satisfactory.

DIVISION OF *TR. BRUCEI*.

Ranvier slide preparations of a growing culture of *Tr. Brucei* were made and studied in the warm room in the same way as in the case of the rat trypanosome culture. Here the study was more difficult, this trypanosome being much more sensitive and readily ceasing all activity under the conditions necessary for the experiment. The small rosettes, which in the case of *Tr. Lewisi* are quiet enough for observation, are here so active that the slide must be continually moved to follow them. The preliminary shortening of the cell before division is not apparent, and for that reason it is more difficult to select suitable cells for observation. It was necessary to select a cell, largely at random, and to watch it continuously for any change. Naturally, large cells might be expected to divide sooner than small ones. As the result of a week spent in the warm room cell-division was twice observed, once in a cell of a small actively traveling rosette, and once in a large single cell attached by its posterior tip to a mass of blood corpuscles.

A slide preparation of an actively growing culture was made at 8:55 A. M. After going over the whole preparation, using the mechanical stage, a rosette of three cells was selected as the most promising, because one of the cells composing it was somewhat larger than either of the others. The large cell, *a*, was overlapped for its posterior one-third by the posterior end of cell *b*, the whips extending free in opposite directions. Cell *c* was wound spirally around the junction of *a* and *b*, its whip extending laterally. The large cell by its active whip overcame the exertions of cell *b* in the other direction, so that the whole rosette traveled constantly in its direction: the third cell, *c*, by its position produced a constant spiral rotation of the rosette. Observation was begun at 9:41 A. M. At 2 P. M. a second whip was seen on cell *a*, very short and close to the old one, although its presence was not certain. At 3 P. M. there was no longer any doubt. A new whip was there and very active. In another half-hour it was much larger and the division had

extended into the cell-body for some distance. The split progressed somewhat farther, but at 4:30 p. m. the motility decreased, and the outlines between the cells became indistinct, and at 5 p. m. all three cells had coalesced into one spherical body, one of the whips, that belonging to cell *b*, still retaining slight activity.

The second observation of division was made upon a single cell (Plate II, Fig. 1). The slide was prepared at 9:30 a. m. The cell was selected on account of its large size and relatively fixed position, and the observation was begun at 10:30 a. m. It was then an unusually thick cell with a long cylindrical posterior tip and a very active flagellum. By its posterior tip it was attached to a mass of blood corpuscles, so there was no progressive motion. The cell is represented in Plate II, Fig. 1. At 6 p. m. a second whip seemed at times to be present on one side of the cell, and at 8:55 p. m. its presence was no longer in doubt (Fig. 2). At 8:52 the next morning this whip was longer, and the division of the cell body could be indefinitely made out by focusing (Fig. 3). An hour later the outline of two cells was distinct and certain, the smaller one lying beneath and anterior to the larger. Both were very active. From the large size of the upper cell a second division was expected to occur soon. The specimen was therefore kept under observation, but all activity ceased at 6 p. m.

A careful examination of the rosettes of *Tr. Brucei* in culture shows them to be made up of cells stuck together in this relation—that is, by their adjacent sides—one cell being slipped forward on the other, as it were. We can conceive therefore, of the rosettes arising by consecutive divisions of this kind. In these rosettes the whips are directed outward, in marked contrast to the culture rosettes of *Tr. Lewisi*. This fact, it may be well to note, corresponds to Schaudinn's observation on the life-cycle of the "leuceocytozoon," in which case the trypanosomes which develop in the gut of the mosquito often agglutinate with their whips directed outward.

THE TOXIN OF *TR. BRUCEI*.

Kanthack, Durham, and Blandford⁹ showed that the blood serum from which the abundant trypanosomes had been removed by filtration had no specific toxic effect, and that enormous doses of blood swarming with the protozoa produced no immediate symptoms. Rich Nagana blood kept for several days until the protozoa had disappeared from it was likewise without toxic effect. From these and other similar experiments they regard the blood as free from specific poison. The successful cultivation of this organism opened up a new way in which to attack this problem. Cultures

which have developed at 25° C. may be readily deprived of living trypanosomes by exposure for five days to a temperature of 34° C. The whole condensation fluid from four flasks ($\frac{3}{4}$ c.c.) treated in this manner was injected into the peritoneal cavity of a white mouse weighing 22 g., without any apparent ill effect. Another mouse of about the same size was injected with $\frac{1}{3}$ c.c. of the condensation fluid from four flasks grown at 25° for fifteen days, and then exposed to 34° for two days. This material still contained actively motile trypanosomes. The animal survived, and when inoculated three weeks later with virulent Nagana blood died in six days. These experiments indicate that *Tr. Brucei* produces no powerful stable toxin.

In the course of some attempts to immunize guinea pigs to this disease we have obtained some evidence of the probable presence of a toxin. The first two or three subcutaneous injections of attenuated cultures have little local effect. A rise in temperature and a loss in weight are observed. After several protective inoculations the quickness of reaction on the part of the animal is increased. The temperature rises within a few hours after the injection, the animal shows signs of pain at the point of inoculation, and often lies down. After a few days a hard eschar forms at this point. This dries and sloughs off after a week or two, and the resulting ulcer heals with scar formation, after which the animal entirely recovers. Subsequent injections produce similar effects. The more or less complete general immunity is then an important factor in this intense local poisoning, and seems to indicate that the toxin becomes active upon the destruction of the trypanosome cells in the body of the living host.

CONJUGATION.

In a recent publication Prowazek²² announces in a few lines the observation of conjugation in *Tr. Brucei* without giving any definite description of the process. Reviewing the question of a sexual process among flagellates in a later publication, he²³ points out the lack of information on this point. Regarding as conjugation only those fertilization processes in which two cells take part, the positive cases are few indeed.

Schaudinn²⁷ describes macrogamete and microgamete forms of

the halteridium, *Hemoproteus noctuæ*, which he reclassifies as a trypanosoma, and the union of these forms to produce an oökinete. Whether this protozoon is properly classified as a trypanosome may well be questioned. At any rate, nothing corresponding to his macrogametes and microgametes or to their precursors, the gametocytes, has ever been described in the cycles of such well-known and representative forms as *Tr. Lewisi* and *Tr. Brucei*.

The conjugation described by Plimmer and Bradford has been regarded as erroneous by the continental workers, and the idea that the smaller chromatin body is merely a base for the flagellum seems to be the most widely accepted view at the present time. However, the observations of Plimmer and Bradford of changes in this body during the so-called agglutination of the trypanosomes are readily confirmed. Stained preparations made of the tail blood of a mouse in a dying condition were found to be particularly rich in these couples. The contact is not merely at the extreme tips, but there is an overlapping of the two ends, and very often such intimate contact that most careful examination at a magnification of three thousand diameters fails to find any line of separation. There appears to be complete fusion and continuity of protoplasm between the cells. One naturally looks carefully for signs of unusual activity on the part of the smaller chromatin body under these circumstances. These bodies frequently lie very close together in these couples, sometimes in such intimate relation that they appear as one body under the greatest magnification. In other cases the two bodies lie close to each other and appear to be connected by a lighter band, as in Plate XVI, Fig. 3. Again a tetrad of four blepharoplasts may be found at the junction, and it is impossible to determine to which cell they may belong (Plate XVI, Fig. 4). Such double trypanosomes are so numerous in the blood of the dying mouse that often three or four of them are to be found in every field of the microscope. They would seem to indicate an actual conjugation effected by changes in the blepharoplast or micronucleus, as Plimmer and Bradford call it. It is impossible to be certain of such a process merely from study of stained preparations, however. The question appears worthy of further study.

In the rat trypanosome the blepharoplast usually has a bilobed appearance in stains made from blood containing agglutinations. Rarely actual fragmentation of this body into equal portions is seen. Exchange of material from one cell to another has not been demonstrated.

CONCLUSIONS.

1. The division of trypanosomes is not exactly longitudinal, but always more or less oblique in direction. The whip does not divide, but a new one is formed in each division. There is only one type of division in the mammalian host and in cultures on blood agar. The process has been observed throughout under the microscope in the case of *Tr. Lewisi* and *Tr. Brucei*.

2. The destruction of trypanosomes in the blood is due to a cytolytic action of the blood plasma rather than to actual phagocytosis. The solution of *Tr. Brucei* in the later stages of Nagana probably sets free the toxin, and thus produces the characteristic symptoms and death. This accounts for the remarkable fact that in some cases the blood may be swarming with the protozoa without marked symptoms on the part of the host, and again another animal may die from the effects of relatively few trypanosomes in the blood. Human trypanosomiasis is an example of the latter class.

3. The culture rosettes of *Tr. Lewisi* have their flagella directed centrally, while those of *Tr. Brucei* are more irregular, but as a rule the whips are on the periphery.

4. The agglutination of trypanosomes is often associated with changes in the smaller chromatin body, suggesting an isogamic conjugation.

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EXPLANATION OF PLATES XI-XVII.

PLATE XI.—DIVISION OF *Tr. LEWISI* IN CULTURE.

- FIG. 1.—Culture rosette; living preparation on Ranvier slide; at 8:25 A. M.
 FIG. 2.—Same at 8:30 A. M. Two cells are in process of division.
 FIG. 3.—8:35 A. M.: Cell *a* has divided, *d* is beginning to shorten.
 FIG. 4.—8:38 A. M.: Cell *d* is shortening rapidly.
 FIG. 5.—Sketched at 8:41 A. M.
 FIG. 6.—8:44 A. M.: The daughter-cells of *a* have elongated and become actively motile. The flagella are not definitely seen.
 FIG. 7.—8:47 A. M.: Cell *e* is shortening.
 FIG. 8.—8:50 A. M.: Cell *b* has become motionless and evidently dead.
 FIG. 9.—8:55 A. M.: Three of the cells are in process of division.
 FIG. 10.—9:10 A. M.: The increasing motility of the lengthening cells has altered the relative positions of the cells.
 FIG. 11.—9:28 A. M.: The motility of the rosette is increasing. Cell *b* continues motionless.
 FIG. 12.—9:39 A. M.: All the daughter-cells have elongated and become active, measuring now about 10μ in length.
 Magnified about 1,400 diameters.

PLATE XII.—DIVISION OF *Tr. BRUCEI* IN CULTURE.

- FIG. 1.—10:30 A. M.: A large cell fixed by its posterior tip.
 FIG. 2.—8:55 P. M.: A second whip has formed.
 FIG. 3.—Next day 8:52 A. M.: The new whip is larger and more active. Division of the protoplasm is apparent on focusing.
 FIG. 4.—10 A. M.: Complete division can be made out by focusing.
 Magnified about 2,000 diameters.

PLATE XIII.—*Tr. LEWISI*.

The microphotographs were all taken, with exception of Fig. 3, Plate XIV, at a magnification of 3,000 diameters, and the reproductions are about three-fourths of the original size.

FIG. 1.—*Tr. Lewisi*, adult stage; blood of rat fifteen days after inoculation.

FIG. 2.—Anomalous form frequently found during the developmental stage; in blood of a rat, six days after inoculation..

FIG. 3.—Cell enlarged preparatory to division; a new whip beginning to grow out from its base near the blepharoplast, the latter not yet divided.

FIG. 4.—Later stage, two distinct whips, blepharoplasts and nuclei, two more whips forming from the elongated blepharoplasts.

PLATE XIV.—*Tr. LEWISI*.

FIG. 1.—Four-cell stage, each cell in process of further division. This specimen has been crushed in spreading so as to destroy the outlines of the cells, a common occurrence.

FIG. 2.—Eight-cell rosette. Note the long original or parent whip on one of the cells. Several of them show a second whip growing out preparatory to a further division.

FIG. 3.—Different stages in the growth of the small to the large, dividing form, in rat blood six days after inoculation. $\times 1,500$, but reduced three-fourths.

FIG. 4.—Rosette of *Tr. Lewisi* in culture on blood agar. Note the central direction of the flagella.

PLATE XV.—*Tr. BRUCEI*.

FIG. 1.—Large and small form. The small cell is the product of a recent division; the large one is preparing to divide.

FIG. 2.—Division into two. Note the projection of one cell posterior to the other. A third whip is forming.

FIG. 3.—Later stage, one cell about to separate.

FIG. 4.—Early stage of division showing independent growth of the new whip. The undulating membrane has been dissolved away by alkaline phosphate solution.

PLATE XVI.—*Tr. BRUCEI*.

FIG. 1.—Agglutination of two similar trypanosomes.

FIG. 2.—Two cells in intimate contact with only one blepharoplast to be made out. Conjugation?

FIG. 3.—Another couple showing the blepharoplasts apparently connected by a chromatin band.

FIG. 4.—A couple with four of the smaller chromatin bodies.

PLATE XVII.—*Tr. BRUCEI*.

FIG. 1.—One kind of the granular form. The nucleus is completely fragmented. Note the long body and relatively short flagellum.

FIG. 2.—Large hyaline trypanosome much less deeply stained than the ordinary type. The nucleus is round.

THE PATHOLOGICAL ANATOMY OF EXPERIMENTAL NAGANA.*

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INTRODUCTION.

ALTHOUGH various workers have described the gross pathological anatomy of trypanosomiasis, few have given any details of the microscopical anatomy of the condition.

In their report on nagana in 1898, Kanthack, Durham, and Blandford¹ state that the number of red blood cells are decreased in this condition; that the hemoglobin is decreased proportionately to the red blood cells; that normoblasts may be found in the circulating blood; and that leucocytosis is frequently present. They found that the lymph glands and spleen were enlarged in rats and mice, and that the liver showed fatty change. These changes were not so marked in the guinea pig, but the bone marrow was frequently hyperplastic, and the spleen, liver, and kidneys contained varying amounts of iron-containing blood pigment.

Plimmer and Bradford² (1899, 1901) added no new microscopical findings. In 1902, Laveran and Mesnil³ published the results of their study on the morphology of *Trypanosoma Brucei* and found that the enlargement of the spleen was due to a congestion without appreciable microscopical changes.

In the same year Voges⁴ published his work on mal de caderas, in which he found an increase of the fluids in all the body cavities. He also found that the lymph glands were frequently enlarged,

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¹ KANTHACK, DURHAM, AND BLANDFORD, *Proc. Roy. Soc.*, 1898, 64, pp. 110-18; translation by NUTTALL in *Hygienische Rundschau*, 1898, 8, p. 1185.

² PLIMMER AND BRADFORD, *Proc. Roy. Soc.*, 1899, 65, pp. 274-81; *Quart. Jour. Micr. Sci.*, 1901, 45, p. 449.

³ LAVERAN AND MESNIL, *Ann. de l'Inst. Past.*, 1902, 16, pp. 1-55.

⁴ VOGES, *Ztschr. f. Hyg.*, 1902, 39, pp. 323-72.

and that the spleen was always increased in size, due in many cases to hyperplasia of the trabeculae. He stated that lungs and heart showed no change; that the liver and kidneys may be enlarged, the latter frequently showing a hemorrhagic nephritis.

In 1903, Elmassian and Migone¹ added another microscopical finding to trypanosomiasis, viz., congestion of the membranes of the brain and the frequent increase in the amount of the subarachnoid fluid.

In the same year Bruce and Nabarro² reported the autopsy findings of a number of cases of sleeping sickness. In general the cases show no signs of acute inflammation, but merely a flattening of the convolutions, an injection of vessels, and an excess of subarachnoid fluid.

A short time after this, Manson³ reported that Mott and Low found the brain of an English woman dying from sleeping sickness to show a perivascular, small cell infiltration.

While the above is not the complete literature of the work, it contains, as far as is possible, all the findings reported by the various workers. In view of the above, and considering the fact that new points might be added to the pathology of experimental nagana, the following work was undertaken.

Some of these animals had been inoculated with pure cultures, others with one or two drops each of infected blood, and others with one or two cubic centimeters of a potassium citrate emulsion of the various organs of an infected animal. The difference of pathological picture presented by the three methods was only one of degree, not one of quality, that resulting from inoculation of pure cultures being least marked in degree.

GROSS PATHOLOGICAL ANATOMY.

General appearance.—Body emaciated. Sclera frequently yellowish. Hair coarse, dry, and rough.

Brain.—Meninges lax, moist; varying degree of congestion; not thickened. Subarachnoid fluid increased. Convolutions of brain slightly flattened; surface smooth, shiny, moist. Vessels of pia injected. Cut surface; moist; vessels congested. Fluid in ventricles slightly increased; choroid plexus injected.

¹ ELMASSIAN AND MIGONE, *Ann. de l'Inst. Past.*, 1903, 17, pp. 241-67.

² BRUCE AND NABARRO, *Reports of the Sleeping Sickness Commission*, No. 1, Aug., 1903 (London: Harrison & Sons); BRUCE, NABARRO, AND GREIG, *ibid.*, No. 4, Nov., 1903.

³ MANSON, *Brit. Med. Jour.*, 1903, 2, p. 1462, Dec. 5.

Mediastinum.—Mediastinal fat small in amount. Thymus increased in size; surrounding fat pinkish in color. Frequently enlarged lymph glands present in mediastinum.

Heart and pericardium.—Pericardium thin, slightly distended; fluid straw color and slightly increased. Epicardium smooth, shiny, moist, not thickened. Myocardium distended with currant jelly clots; walls slightly thickened, otherwise negative. Endocardium clear, shiny, moist.

Lungs.—Vary in color from light pink to dark red in a given lung. As a whole the lung floats on water, but frequently parts of lung would sink. Such parts would be dark in color, airless, and give rich exudate on pressure from alveoli. Bronchi negative. Vessels congested.

Spleen.—Greatly enlarged. Edges rounded, and organ as a whole curved upon hilus. Surface smooth for most part, but frequently would have small fibrin tags attached. Peritoneum over spleen thickened. Organ firm; consistency increased. Cut surface: Follicles increased in size; stand out as white or grayish spots. Pulp reticulum and trabeculae increased in size and amount. Pulp flow very slight.

Liver.—Slightly enlarged. Surface smooth, shiny, moist. Dark red in color. Consistency normal. Cut surface bleeds freely. Central vein distended. Central bone of liver lobule slightly yellow in color. Glisson's capsule not increased in rats or in mice, but slightly increased in guinea pigs.

Intestines.—Slightly distended. Serosa smooth, shiny, moist. Walls negative on section. Pancreas negative.

Kidneys.—Slightly increased in size, red in color. Consistency slightly increased. Fatty capsule thin. Fibrous capsule not thickened; strips easily. Cut surface of kidney moist. Glomeruli stand out as small red points. Labyrinth slightly enlarged; all vessels congested. Adrenals negative.

Lymph glands.—Retroperitoneal, enlarged, pinkish, moderately firm.

External genitals.—Slightly edematous.

Ovaries or testicles.—Negative, except for slight edema and congestion.

Bone marrow.—Sternum and femur hyperplastic. Fatty marrow of femur replaced by varying amounts of lymphoid marrow.

MICROSCOPICAL ANATOMY.

Spleen.—Certain changes were constant in every section from every animal examined, but the degree of this change varied greatly. This variation seemed to have no relation with either the mode of injection, the injected material, or the time elapsing between the injection and death.

The follicles are enlarged in every section. The enlargement of a greater number of these follicles is due largely to an increase in the number of a certain form of cell. These cells are large, round or oval, somewhat larger than a polymorphonuclear; they possess a coarsely granular protoplasm, surrounding a round or oval, deeply staining nucleus, which is excentrically situated and whose chromatin is slightly bunched. With Ehrlich's triacid mixture the protoplasm of some of these cells shows fine or coarse granules staining a reddish purple. With eosin and methylene blue combination the greater number of these cells are seen to contain coarse granules staining a deep blue. Other enlarged follicles show two distinct zones—an inner lightly

staining, and an outer deeply staining zone. The inner zone is composed of cells whose protoplasm stains faintly, and whose nuclei are centrally placed and stain lightly. The outer zone is composed of cells resembling those first described above. Between these two zones transition forms are sometimes seen—cells which are smaller than the central cells, their chromatin more bunched, and their protoplasm showing scattered granules. The other cells which are found in the enlarged follicles are about the size of a red blood cell, contain practically no protoplasm, and possess a round, deeply staining, compact nucleus.

What has been said about the fact that the changes in the follicles have no relation to the mode of infection, etc., is also true of the pulp. The pulp in every case is congested. Sometimes this congestion may be central; at others it is seen only at the periphery of the organ. In the older cases the stroma is increased in a varying degree. The cells vary also. Cells resembling those of the first type described above are increased in a varying amount everywhere throughout the pulp. Scattered among them are small and large lymphocytes, and a few nucleated red blood cells. In some sections the lymphocytes are bunched in masses of ten to thirty. In other sections the stroma was increased greatly, and the cells seem to be arranged in cords along definite trabeculae of pulp stroma.

Varying amounts of iron-containing pigment were present in every section of spleen, and in the case of this pigment a definite relation seemed to exist between the amount present and the time elapsing between the injection of the infected material and the time of death.

In spleens from animals injected with artificially grown trypanosomata the amount of iron-containing pigment which was deposited increased up to the eighteenth day; the spleens of animals dying on the twenty-first day were less; those of the twenty-second, still less; those of the twenty-third, slightly increased over the twenty-second; those of the twenty-fourth and fifth are increased to about the same amount as those of eighteen days. In spleens of animals dying on the twenty-ninth day the amount of iron-containing pigment is almost normal, but the spleens of the animals dying on the thirty-sixth day contain as much as those dying on the eighteenth day.

The increase in the amount of iron-containing pigment in the spleen of animals injected with the organ suspensions is seen earlier. The first great increase is on the eleventh day; falls to practically normal on the twenty-seventh; rises on the twenty-sixth to a greater amount than on the eleventh, to fall to normal on the twenty-eighth, where it remains till the sixty-first day, when it rises slightly, and remains so until the one hundred and fifth day.

Spleens from animals injected with infected blood show the greatest amount of iron-containing pigment. On the fifteenth day the first increase is at its height; falls on the nineteenth to practically normal; is increased again on the thirtieth to the thirty-sixth; falls again on about the fortieth, to rise again on the ninety-second.

Lymph glands.—All lymph glands show a great increase in the number of lymphocytes, accompanied by a greater or less hyperplasia of the stroma. In some glands taken from an animal which had been injected with one drop of

infected blood, and which lived for ninety two days, a large number of mono nucleated white blood cells are present. Some of these cells contain coarse oxyphile, others fine oxyphile cells. In the sinuses of some glands large round or oval hyaline cells are present which contain remains of red blood cells or granules of iron-containing pigment. The stroma of these glands are markedly hyperplastic.

Lymph glands taken from animals which were inoculated with cultures show no changes. Those from animals injected with organ suspensions show similar changes to the ninety-two-day blood inoculation animal, but the changes are less in degree.

Thymus.—The increase in size of the thymus is due to a simple hyperplasia of the lymphoid elements and the stroma.

Liver.—The liver cells at all stages show a cloudy swelling, varying in degree from the earliest to a stage where the outline of the liver cell is very poorly made out, and the cords of cells are a granular mass with poorly staining nuclei. Frequently these areas contain large unmbers of adult trypanosomata.

Fatty degeneration accompanies this cloudy swelling, but seems to vary in degree according to the method of inoculation. In those animals which were injected with cultures a small amount of fatty degeneration is seen in the livers of animals dying on the fifteenth day; those dying on the eighteenth day show a slight increase over the fifteenth-day animal, but animals dying later show no increase. Some parenchymatous cells of the livers from animals injected with 1-2 c.c. of the citrate suspension of organs of infected animals, and which died on the tenth day, show a moderate number of large fat drops. The protoplasm of practically all other cells is composed of highly refractive droplets. The cells from livers of animals dying on the fifteenth, twenty-sixth, and seventy-first days show less fatty degeneration; those of the twenty-second and twenty-eighth show about the same amount of fatty degeneration as those dying on the tenth; while those dying on the one hundred and fifteenth day show practically no change.

Parenchymatous cells from livers of animals inoculated with one drop of infected blood show the greatest change. Cells from the liver of an animal dying on the ninth day show a moderate fatty degeneration; those dying on the thirteenth to fifteenth, marked; those on the nineteenth, thirty-seventh, and ninety-second, less. Aside from cloudy swelling and fatty degeneration, the livers show nothing pathological, except an occasional variable leucocytosis.

In those animals which were inoculated with one drop of infected blood, and which died on the fourteenth day, the livers show a relatively large number of capillary endothelial cells withholding iron-containing pigment, but the liver cells are free. The liver or endothelial cells of all other animals show no iron-containing pigment.

Lungs.—The pulmonary vessels of all lungs coming to autopsy are greatly distended. The alveolar capillaries are so dilated and filled with blood that it is frequently difficult to outline the individual cells. Between the blood cells varying numbers of adult trypanosomata are present. In some areas the alveoli contain a granular exude inclosing desquamated epithelial cells. In

other areas the alveolar spaces are compressed, and their walls are folded upon themselves. In other areas some alveoli contain red blood cells, leucocytes, a small amount of stringy or granular fibrin, and desquamated epithelium. Some bronchi show a marked mucous degeneration.

Heart, adrenals, salivary glands, pancreas, testis, ovary, are negative except for a varying amount of acute congestion.

From the foregoing pathological histology it will be seen that the changes produced by *Trypanosoma Brucei* are those of an intoxication. The spleen shows a congestion, accompanied by hyperplasia of the pulp reticulum in some areas, and by a varying degree of hyperplasia of cells corresponding in morphological characteristics to myelocytes. The greater number of cells of the normal spleen are lymphocytes and the follicles are well outlined, but in all the spleens taken from animals infected with *Trypanosoma Brucei* the great enlargement is due to an increase in the stroma of the pulp, accompanied by an enlargement of the follicles. This enlargement of the follicles is due to an increase in the number of forms of cells. As mentioned above, the central zone of the follicles, which is composed largely of endothelial cells, is enlarged. Outside this is another zone, consisting of mononuclear cells composed of a coarsely granular protoplasm surrounding a single, deeply staining nucleus. The protoplasm of these cells contains both fine and coarse oxyphile granules. In other words, these cells resemble morphologically the cells designated as myelocytes in man. Between these two forms of cells are transition forms which are present in the boundary between the two zones, so that it is possible to say in many sections that the myelocyte develops from an endothelial cell.

Accompanying this marked hyperplasia and new formation of myelocytes, few red blood cells are seen and scattering giant cells resembling those of the bone marrow. Many of these cells are seen to be developing from endothelial cells of the pulp. These cells have a single, lobulated, deeply staining nucleus, surrounded by a finely granular protoplasm.

Another important change in the spleen is the deposit of iron-containing pigment—hemosiderin. From the description it can be seen that the amount of iron-containing pigment in the

spleen increases until the fifteenth day: then the amount is less for eight to ten days, when there is another increase, followed by another decrease, which in turn is followed by another increase.

The explanation for this seems to be that the body energies are able by certain explosive efforts to overcome the agent which is causing the destruction of red blood cells, which is shown by a decrease in the amount of hemosiderin in the spleen. These energies are unable completely to destroy these agents, and the animal eventually succumbs.

What the destructive agent may be it is difficult to say. That the *Trypanosomata* themselves do not attack the cells is clear beyond doubt: consequently, it is quite probable that they excrete some substance which is hemolytic, and the end result of this process is the hemosiderin found in the spleen.

On the part of the other hemopoietic organs, the marrow of the long bones becomes hyperplastic, as do the lymph glands. Here it may be added that lymph glands taken from animals whose spleen shows the greatest hemosiderin contain a few hyaline cells with remains of red blood cells.

Plimmer and Bradford, and Laveran and Mesnil, have suggested that *Trypanosoma Brucei* produces its effects by means of some toxin which it forms, but these workers could not demonstrate the toxin. Nevertheless, from the histological findings given above, it seems very conclusive that the pathological changes are due to a toxin.

To sum up briefly the pathology of experimental nagana, it may be said that this disease is caused by *Trypanosoma Brucei*, which produces a mild intoxication acting chiefly on the blood and blood-forming organs.

I wish to thank Dr. Novy, Dr. Warthin, and Dr. MacNeal for the patience shown, and the kindly assistance and useful aid given me throughout this work.

THE BLOOD IN MEASLES.*

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LITERATURE.

THE literature on the blood in measles is not extensive. The earlier reports relate chiefly to the enumeration of the white and red cells, and to the estimation of the hemaglobin, and it is only in recent years that thorough studies have been made by Türk, Reckzeh, and others.

The first observations date back to 1887, when Widowitz, estimating the hemaglobin in various diseases, found in eighteen cases of measles a moderate decrease at defervescence, followed by an increase during convalescence. Hayem (1889) recorded 10,000 and 14,000 white cells in two cases, the second complicated by angina. The red cells were only slightly reduced, seldom as many as 500,000. Pée (1890) examined five cases by a rather unreliable method. In four he found normal or diminished leucocytes; in the fifth, a count of 15,000, which he attributed to the puerperium. He found the large mononuclear cells increased. Pick (in the same year) reported one case with no leucocytes.

Rieder (1892) studied the blood in eight cases, finding normal and subnormal counts, the lowest 2,700. In one apparently uncomplicated case he found 15,000 on the fourth day of the rash, in a three-year-old child. A complication by bronchopneumonia showed 15,500. Rieder was the first to call attention to the possible value of the lack of leucocytes in differentiating between measles and scarlatina.

Rille (1892), on examining twenty cases, reported in part a slight increase in eosins and marked increase in lymphocytes, in part normal findings. He did not state the period of the disease. Felsenthal (1892) found no leucocytosis in eight cases, but rather a diminished number of leucocytes, the proportion of white cells to reds with normal red count being 1:1000. The neutrophiles

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formed the bulk of the leucocytes, the mononuclear cells varying from 10 to 20 per cent., and the transitional from 5 to 8 per cent. He found the eosins not exceeding 1 per cent., and stated his belief that this with the absence of leucocytosis was of value in the diagnosis from scarlatina. Zappert (1893) found during the fever no rise of leucocytes and of eosins, but in convalescence a rise of 3 per cent. of the latter.

Sobotka then (1893) made the interesting observation that during the period of incubation of various acute infectious diseases—namely, pneumonia, scarlatina, varicella, small-pox, and measles—there occurs a leucocytosis, which persists in pneumonia and scarlatina throughout the acute stage, but in measles, small-pox, and varicella gives place to normal or subnormal counts shortly before the outbreak of the disease. He gave no figures to illustrate this, except in the case of small-pox. Klein (1893) noted an increase in the large mononuclear cells.

Türk (1898) made a very complete study of the blood in three cases, all adults. He found in all a diminution of the leucocytes during the rash, with a rapid return to normal during convalescence. The polynuclear neutrophiles were at first relatively, but not absolutely, increased, sinking later to normal or subnormal figures. Eosins were, during the eruption, normal or diminished, later increasing to normal or somewhat above (4.7 per cent.). The large mononuclear and transitional cells were increased during the later stages. The red cells and hemoglobin showed no changes worth mentioning. The blood plates were roughly estimated as scanty to normal during the eruption. Fibrin, as estimated by the amount of network in the fresh specimen, was scarce, sometimes plainly decreased. Türk drew the following conclusions: Uncomplicated measles never gives rise to leucocytosis, but rather to leucopenia; a leucocytosis, if present, points to a complication; the specific bronchitis of measles gives rise to no leucocytosis, except in the presence of a mixed infection; leucopenia or repeated normal counts point to measles or against scarlatina, while marked increase in eosins, especially with leucocytosis, is in favor of scarlatina.

Cabot (1898) gave figures from seven cases with normal and

subnormal white counts. In two cases of rubella the counts were 6,000 and 8,000 respectively.

Combe (1899) gave a brief abstract of the work of his pupil Renaud, whose monograph appeared the next year. Renaud examined four cases during incubation and found in all a polynuclear leucocytosis, setting in soon after infection occurred, and reaching its maximum six days before the appearance of the rash. The white count rose gradually to its maximum, then declined, reaching normal in the latter part of the stage of invasion. His maximum counts were 34,000, 17,000, 13,600, 17,300. He also studied the blood in twenty-three other cases of measles, all children. In uncomplicated cases he found constantly leucopenia, with lowest counts about twenty-four hours after the appearance of the rash. He recorded a count of 1,300 in the case of a two-year-old child on the third day of the rash; death five days later from bronchopneumonia. The leucocytes reached normal numbers from one to five days after the disappearance of the rash. The presence of leucocytosis always pointed to a complication, and was in some cases the first sign of the same. The absence of leucocytosis with a complication, or a marked and long-continued leucopenia in uncomplicated cases, was of bad prognostic import. He noted that a high normal count during the period when there should be leucopenia may have the same significance as a leucocytosis at a later stage. He concluded that the bronchitis and conjunctivitis of measles give rise to leucocytosis. It should be remembered, however, that his method of estimating the leucocytes (he counted only 256 of the small squares of a single Thomas-Zeiss chamber) would allow of a considerable error.

Renaud made differential counts in most of his cases, but only one or two in each. He found the neutrophiles increased, both relatively and absolutely, during the leucocytosis of incubation; at the time of the rash they decreased, contrary to the observations of Türk and Reckzeh. This may perhaps be accounted for by the fact that he placed the normal percentage in children too high. Eosins were in normal proportion until the outbreak of the eruption, when they disappeared entirely, to return during convalescence. Myelocytes were noted during the period of exanthem in

considerable numbers. Lymphocytes during the incubation were relatively decreased, absolutely increased, during the eruption, relatively increased. The red cells showed no changes. Renaud concluded that the presence of a leucocytosis in a person exposed to the contagion of measles showed that the disease has been contracted, provided that other causes of leucocytosis could be excluded. This, he thought, would be of great practical advantage in prophylaxis, for it is well known that by the time Koplik spots or other signs of measles have appeared, isolation is no longer effective.

Caccia (1900) reported the findings in twenty-three cases, of which fourteen were uncomplicated. In all of the latter leucopenia was present, being greatest on the first day of the rash and reaching normal about seven days later. Bronchopneumonia or diphtheria occurring during the rash gave normal or slightly increased counts, but not a marked leucocytosis, which is to be expected if this complication occurs during defervescence. Measles in a case of tuberculosis diminished a pre-existing leucocytosis, which reappeared during convalescence. His examinations of stained specimens were incomplete.

In his text-book on the examination of the blood, Ewing (1901) mentions three cases of his own occurring in malarial subjects in which leucocytosis was absent.

Reckzeh (1902) made repeated examinations in children in ten cases. His counts agree with those of other observers, showing leucopenia at the acme, increasing counts at the paling of the rash. He found no relation between the severity of the process and the lowness of the count. Frequently leucocytosis occurred during convalescence, most often due to inflamed glands, sometimes without assignable cause. He claimed that the bronchitis of measles gives rise to leucocytosis, but his tables hardly bear out this assertion. Cervical adenitis and otitis media caused leucocytosis; a case with broncho pneumonia had 8,000 white cells. Acute nephritis gave no leucocytosis. His differential counts agreed with those of Türk, except that he found the eosins constantly diminished during the rash.

The latest work on the subject is that of Plantenga (1903),

who made counts in twenty-nine cases of measles and ten cases of rubella, or German measles, all in children. In eight cases examined during the period of incubation he found a leucocytosis ranging from 20,000 to 33,000, due to increase of the neutrophiles and lasting till two days before the outbreak of the eruption. He thus confirms the observations of Sobotka and Renaud. He observed the usual leucopenia during the eruptive period. In rubella he recorded the same blood findings as in measles in every respect, including the leucocytosis during incubation, with the succeeding leucopenia. His differential counts were done in a liquid medium in the Thoma-Zeiss chamber, and therefore are hardly worthy of consideration. No clinical accounts of his cases were given.

It remains to mention certain attempts to find the etiological factor of measles in the blood. Giarre and Combe made cultures from the blood, with negative results. Barbier in ten cases reported cultures either negative or contaminated by the skin. Weber (1896) discovered protozoa in the blood during the eruptive stages of measles, scarlatina, and small-pox; his observations have not yet been confirmed by others.

PERSONAL OBSERVATIONS.

In the spring and summer of 1902 I made repeated examinations of the blood in twenty-eight hospital cases of measles, with the hope of rendering our knowledge of the blood picture more complete and of learning perhaps something of practical importance.

As the methods employed in this kind of work are of great importance, I shall describe them somewhat in detail. The white counts were done in a Thoma-Zeiss chamber, as modified by Elzholz; this permits of counting nine large squares with a single drop of fluid, instead of only one, as in the original chamber. At least two hundred white cells were counted at every examination; and sometimes as many as eight hundred. The blood was obtained from the lobe of the ear, with all the usual precautions against error, and a 1 per cent. solution of acetic acid, colored, as suggested by Türk, with gentian violet, was employed

as the diluting fluid. The dilution was usually 1:20, sometimes 1:10. Owing to lack of time, it was often necessary to make the counts after meals, and so in some cases the element of digestion leucocytosis must be reckoned with. The patients were all on liquid diet until after defervescence; this, as is well known, gives rise to no digestion leucocytosis. In convalescence they were on a mixed diet, consisting of a breakfast of carbohydrates at 8 A. M.; dinner, with meat and vegetables, at noon; and a light supper at 5:30 P. M. As the hour at which the count was made is always given, it is possible to make due allowance for the effects of digestion. The red counts were done by the Thoma-Zeiss method, using Gowers's solution and a dilution of 1:200; usually 1,500 red cells were counted. The hemaglobin was estimated by the Tallquist method, which, allowing an error of about 10 per cent., proved satisfactory in my hands. I do not consider it sufficiently accurate for scientific purposes.

In making the differential counts, the classification of Ehrlich was followed, as being that most generally in use. The stain employed was Jenner's, as modified by Leishman (polychrome methylene blue and eosin in one solution). This gives clearer pictures than the Ehrlich triple stain, especially as to the nuclei and the mast cells, and is at the same time easier and surer of application. It was attempted to distinguish between the large lymphocytes and the large mononuclear cells, but this was found too difficult to be of practical value, there being many cells which might be called one or the other according to the standard of the observer. I therefore included the large lymphocytes, large mononuclear, and transitional forms in one group. Four hundred cells were counted in each instance. The blood-plates were not counted, but roughly estimated as normal, increased, or diminished in number.

The patients being hospital cases and many of them children, the histories were often incomplete. In such instances I have assumed that the rash appears on the fourth day of the disease. The treatment was purely symptomatic, and in most cases no drugs were used, except a simple cough mixture. Patients were kept in bed until several days after defervescence.

Before considering the cases, it remains to discuss briefly normal blood. For fasting adults the normal white count may be placed at 7,500, with extreme variations between 5,000 and 10,000. After a meal containing proteids a rise of from 2,000 to 4,000 may be expected, but not always. The polynuclear neutrophiles will be found to make up 60-75 per cent.; the lymphocytes, 18-25 per cent.; the large mononuclear and transitional, 2-4 per cent.; eosins, 1-4 per cent.; mast cells, 0- $\frac{1}{2}$ per cent.

In young children and infants the white count is higher than in adults, and leucocytosis more easily produced and higher in proportion to the cause. For infants the normal number varies from 10,000 to 15,000, with an increase at times to 20,000 or above from slight or unassignable causes. In the second year the count is about 12,000, falling gradually until it reaches that normal for adults at about the tenth year. Children show a marked decrease in polynuclear neutrophiles and an increase in lymphocytes, as compared with adults; Gundobin, for instance, found in normal infants during the first year 28-40 per cent. neutrophiles and 50-60 per cent. lymphocytes; in the second year, about the same relations; in the third year, 55 per cent. neutrophiles and 39 per cent. lymphocytes; after that, a gradual change until adult proportions are reached at about the tenth year. Digestion leucocytosis is the same as, or somewhat greater than, in the adult.

Furthermore, the blood may be modified by pre-existing chronic disease; as, for example, an increase in the lymphocytes may be the result of rickets or "scrofulous affections," or even of simple debility, as shown by Cabot. All this emphasizes the necessity of individualizing before drawing conclusions from blood examinations, as pointed out by Türk, and taking all the factors into consideration. A white or differential count which is normal for one individual may be pathological for another.

CASE I.

DIAGNOSIS — MEASLES.

Female, age twenty-five years. Past history: Mumps and scarlatina in childhood.

Present illness: Onset May 8, with nausea, headache, cough, and rash.

Physical examination, May 10: Well nourished. Considerable prostration; vomiting; conjunctiva and throat congested; Koplik spots on both cheeks; eruption on roof of mouth; typical maculopapular rash over body, confluent on face and chest. A few râles: systolic murmur at apex.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
May 10, 5 P. M. . . .	6	103.2	4,600	85.50	8.75	5.50	0	0.25	—	Hg. 85% red cells 5,208,000.
May 13.	9	99.3	6,000	49.75	32.75	16.50	0.75	0.25	Sl. —	Rash fading; desquamation on face.
May 16.	12	98.5	9,200	68.00	22.25	8.00	1.25	0.50	—	Some remains of rash; convalescent.
May 19, 12 M.	15	98.7	11,400	80.00	15.00	4.00	0.50	0.50	N.	Rash gone; desquamation present.
May 23, 3:30 P. M.	19	98.8	11,400	79.25	17.25	2.75	0.50	0.25	N.	19th. Hg. 85%; red cells 4,996,000.
May 29, 4:30 P. M.	25	10,800	71.50	20.75	6.75	0	1.00	N.	Heart normal; very slight desquamation; discharged well May 30.

CASE II.

DIAGNOSIS — MEASLES.

Male, age twenty-seven years. Past history: Whooping cough in childhood.

Present illness: Onset May 7, with fever, coryza, pains in limbs, vomiting. Cough three days. Rash appeared today.

Physical examination, May 13: Well developed and nourished. Conjunctivitis; Koplik spots; maculo papular rash, becoming confluent on face, discrete on trunk, absent on legs; eruption on roof of mouth. Soft systolic murmur: few râles.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
May 13, 5 P. M...	6	101.0	8,200	79.40	11.20	9.20	0.20	0		500 cells counted.
May 16, 5 P. M...	9	99.5	6,700	64.00	24.50	7.25	4.00	0.25	Sl. -	Vomiting and diarrhea on 14th; petechae on abdomen and back today.
May 19, 12:30 P. M.	12	98.1	10,100	77.75	15.25	4.75	2.00	0.25	N.	Comfortable; petechae fading; no râles; desquamation present.
May 22, 6 P. M...	15	97.8	10,500	70.50	25.00	2.25	2.00	0.25	N.	Up; good convalescence; hg. 90%; red cells 4,796,000.
May 26, 11:30 A. M.	19	97.6	14,900	80.25	16.00	2.25	1.50	0	N.	Nothing objective to account for leucocytosis
May 29, 1 P. M...	22	99.8	9,400	75.75	14.75	7.00	2.00	0.50	+	Still slight desquamation on hands.
June 4, 3:30 P. M.	28	98.5	9,200	68.50	24.50	1.50	5.00	0.50	-	Heart normal; discharged well.

CASE III.

DIAGNOSIS — MEASLES.

Male, age twenty-one years. No history obtained.

Physical examination, May 5: Well developed and nourished. Eyes suffused: numerous Koplik's; mouth and throat red; characteristic maculopapular eruption, confluent on face, discrete on trunk and extremities. Soft systolic murmur at apex; numerous coarse râles.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
May 5, 3:30 P. M.	5±	102.3	3,000	84.00	9.50	6.25	0	0.25	-	
May 7, 8 A. M...	7	98.7	3,900	63.25	20.75	11.25	4.75	0	-	Crisis today; 1 myelocyte seen; rash fading.
May 9, 8 A. M...	9	98.0	5,100	50.25	37.50	9.25	2.75	0.25	-	Rash gone except where it has been petechial; no râles.
May 12, 9 A. M...	12	97.9	5,900	63.50	27.25	7.75	1.00	0.50	-	
May 15, 11:30 A. M.	15	98.4	6,100	71.75	16.00	10.75	1.25	0.25	N.	Normal convalescence
May 18, 8:30 A. M.	18	6,900	72.50	19.00	6.75	1.00	0.75	N.	Hg. 75%.
May 23, 10:30 A. M.	23	6,300	69.50	22.00	7.00	1.25	0.25	-	Heart normal; discharged well May 24.

CASE IV.

DIAGNOSIS — MEASLES.

Male, age about twenty-five years. No history obtained.

Physical examination, May 15: Well developed and nourished. Conjunctivae red: mouth and throat injected; Koplik spots present; macular rash on roof of mouth: the characteristic rash of measles is present, confluent on the face, discrete elsewhere. Systolic murmur and a few râles.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
May 6	5 ±	105.6	5,900	81.25	14.00	4.50	0	0.25	N.	Hg. 95%; red cells 5,168,000; much prostration; cold bath at 9 P. M. and 1 A. M.
May 18, 3:30 P. M.	7	100.8	7,500	70.00	20.00	5.50	4.50	0	—	Yesterday rash very brilliant, fading today; desquamation beginning; 1 myelocyte seen.
May 20, 5 P. M. . .	9	98.3	7,100	52.75	36.50	6.75	3.50	0.50	sl. —	Rash gone except petechiae; few râles; 1 myelocyte seen.
May 24, 3 P. M. . .	13	98.4	13,200	74.00	21.50	1.75	2.50	0.25	+	Hg. 90%; reds 5,876,000; hot day, sweating; no râles; convalescent.
May 29, 2 P. M. . .	18	98.3	10,200	78.00	18.50	1.50	2.00	0	N.	Discharged well.
June 5, 8 A. M. . .	25	11,900	82.00	12.00	4.25	1.25	0.50	—	

CASE V.

DIAGNOSIS — MEASLES.

Male, age eighteen months; transferred from Massachusetts General Hospital, where he had been treated for pneumonia.

Physical examination, May 16: Pale child with well-marked rachitis. Conjunctivae red; slight coryza; Koplik spot on right cheek; rash on hard palate: fading eruption of macules and papules characteristic of measles, confluent on the face, elsewhere discrete; beginning desquamation. Systolic murmur. A few scattered moist râles: discharging ischiorectal abscess.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	White Cells	Blood Plates	Remarks
May 17.....	6±	102.7	5,200	40.75	53.50	4.50	1.25	0	—	Hg. 60%; reds 4,409,000; eruption fading; small abscess on buttock incised and drained; 4 myelocytes seen; achromia.
May 19, 5 P. M. . .	8	103.6	9,300	46.50	45.25	5.25	3.00	0	N.	Rash nearly gone; rapid respiration; numerous râles; 4 normoblasts; achromia.
May 21, 10:30 A. M.	10	98.8	10,100	48.00	41.00	6.25	4.50	0.25	+	Condition good; crisis; localized bronchitis right apex; achromia
May 24, 5:30 P. M.	13	99.3	20,100	69.50	20.50	9.00	0.50	0.50	+	poikilocytosis; 4 normoblasts; 2 myelocytes; 2 megaloblasts
May 29, 6:30 P. M.	18	11,200	Abscess incised yesterday; June 15, whites 9,700; reds 5,188,000; Hg. 75%; discharged well June 16.

CASE VI.

DIAGNOSIS — MEASLES.

Male, age five years. Past history: Entered hospital April 16 with scarlatina. On May 12 the temperature rose to 103°. Normal next day. Koplik spots were seen on the 14th, and the rash of measles on the 17th.

Physical examination, May 17: Well-developed, fairly nourished boy, with discharge from both ears (scarlatina). Bright maculopapular eruption of measles, chiefly on face, sparse on body and legs.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
May 17.....	5	104.0	9,000	71.25	24.00	4.75	0	0	—	Hg. 55%; reds 3,256,000; 3 myelocytes; achromia.
May 19, 4:30 P. M.	7	100.2	9,200	40.75	50.75	7.25	1.25	0	N.	Mild case; eruption already gone; euphoria; 5 myelocytes.
May 22, 5:30 P. M.	10	98.8	10,400	36.75	48.50	9.25	4.75	0.75	N.	General desquamation; reds show achromia and poikilocytosis.
May 26, 5 P. M. . .	14	98.6	13,300	39.75	52.25	6.25	1.25	0.50	N.	Digestion leucocytosis; 1 myelocyte.
May 31, 3 P. M. . .	19	98.8	11,700	45.25	47.75	5.00	0.50	1.50	+	Discharge from ears ceased; good convalescence.
June 6, 8 A. M. . .	25	98.7	8,500	53.25	40.50	5.00	0.50	0.75	N.	Hg. 65%; reds 4,852,000. Discharged well June 8.

CASE VII.

DIAGNOSIS — MEASLES.

Female, age 25 years. Past history: Measles, mumps, varicella, and scarlatina in childhood.

On May 28 the present illness began, with pains in limbs and cough; later, soreness of eyes. Two days later the rash broke out.

Physical examination, May 31: Well developed and nourished. Eyes suffused and red; macular rash on hard palate, but no Koplik spots: on the body a typical maculopapular rash, most abundant on face, sparse on extremities. Soft systolic murmur at apex; occasional dry r le.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
June 1, 10 A. M..	4	101.0	6,700	74.50	20.25	4.75	0.25	0.25	—	Hg. 85%; rash still bright.
June 3.....	6	98.5	53.75	30.75	11.50	3.50	0.50	N.	1 myelocyte.
June 4, 3:30 P. M.	7	99.0	11,200	50.00	37.50	9.25	2.75	0.50	N.	Rash gone; no desquamation.
June 7, 9 A. M..	10	98.2	13,100	58.00	31.00	7.25	3.25	0.50	—	Slight coryza; no cause for leucocytosis apparent.
June 10, 10 A. M..	13	98.5	14,800	67.75	22.50	5.25	4.50	0	N.	Slight desquamation on feet.
June 13, 11 A. M..	16	98.5	11,600	71.25	19.75	4.75	3.50	0.75	N.	Discharged well June 19.
June 18, 10 A. M..	21	12,000	54.50	37.00	5.00	2.75	0.75	

CASE VIII.

DIAGNOSIS — MEASLES.

Male, age twenty-one years. Measles three years ago. Present illness came on May 20, with cough and fever.

Physical examination, May 23: Well developed and nourished. Conjunctivae; mouth and throat reddened; Koplik spots on both cheeks; macular eruption on hard palate; bright-colored blotchy eruption over entire body, thickest on face. Heart negative; a few dry râles. Enlarged glands under angles of jaw. Temperature 103.4°.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
May 24, 10 A. M..	5	98.5	5,600	45.75	41.25	10.75	2.25	0	—	Hg. 95%; reds 5,096,000; rash beginning to fade on extremities; crisis.
May 26, 1 P. M..	7	98.2	8,100	53.25	34.50	8.75	3.50	0	—	Rash still evident.
May 29, 1:30 P. M.	10	98.4	13,600	77.25	13.25	6.00	3.00	0.50	N.	Probably digestion leucocytosis; rash gone; desquamation.
June 2, 7 P. M..	14	97.5	14,900	71.50	16.00	8.50	3.50	0.50	N.	Digestion leucocytosis; convalescent.
June 6, 9 A. M....	18	98.9	6,200	62.25	20.50	14.25	2.75	0.25	N.	
June 11, 10 A. M..	23	98.4	7,600	63.00	28.75	5.25	2.50	0.50	—	Discharged well June 13.

CASE IX.

DIAGNOSIS — MEASLES.

Female, age eight years. Diphtheria two and one-half years ago, followed by stenosis of larynx, in consequence of which tracheotomy was performed and later gradual dilatation with sounds.

Present illness began May 18, with cough, lachrymation, coryza, and dyspnea.

Physical examination, May 23: Well developed and nourished. Suffusion of eyes: mouth and throat red: a few Koplik spots on cheeks; macular eruption on hard palate; discrete reddish macules and papules scattered over face, trunk, and extremities. Heart negative: scattered râles. Laryngeal stenosis, requiring insertion of tracheotomy tube through the old scar; this happens often with her in "catching cold," as it has been impossible to dilate the stenosed part sufficiently.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
May 24, 12:30 P. M.	6	101.0	9,400	83.75	13.50	2.75	0	0	—	Hg. 80%; reds 5,309,000; rash still present on 25th; respiration easy.
May 26, 5:30 P. M.	8	98.8	9,000	36.00	52.75	8.50	2.50	0.25	—	Crisis yesterday: rash gone; euphoria.
May 29, 4 P. M. . .	11	98.5	8,700	60.75	29.50	6.00	3.50	0.25	N.	Desquamation: still a few dry râles; 1 myelocyte.
June 1, 11 A. M. . .	14	98.7	16,200	73.50	17.25	5.75	2.75	0.75	N.	Up; no cause found for leucocytosis; no symptoms.
June 5, 7:30 A. M.	18	98.1	8,100	62.50	22.75	9.25	4.75	0.75	N.	Doing well.
June 9, 9 A. M. . .	22	98.5	6,900	62.75	28.50	6.00	2.25	0.50	N.	Discharged well June 10.

CASE X.

DIAGNOSIS — MEASLES.

Male, age twenty-two years. Mumps four years ago: rheumatism five years ago. Present illness began May 23, with cough, sneezing, and headache.

Physical examination, May 27: Fairly developed and nourished. Eyes suffused; mouth and throat reddened: macular eruption on hard palate; characteristic maculopapular rash on body, confluent on the face. Heart and lungs negative.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
May 27, 5:30 P. M.	5	101.0	6,600	80.75	13.00	5.50	0.50	0.25	—	Rash fading; face beginning to desquamate; feeling better.
May 29, 3 P. M.	7	98.8	6,700	40.75	40.50	11.00	7.75	0	N.	
May 31, 12:30 P. M.	9	98.5	5,600	55.50	29.00	10.25	4.25	1.00	+	Well-marked desquamation.
June 2, 5:30 P. M.	11	97.7	9,700	74.00	17.50	6.00	2.25	0.25	Sl. —	Normal convalescence.
June 5, 8:30 A. M.	14	98.2	7,300	62.00	23.50	11.00	3.00	0.50	—	
June 8, 10:30 A. M.	17	98.2	5,400	56.75	28.75	11.25	3.00	0.25	N.	
June 13, 10 A. M.	22	98.2	8,300	72.50	19.00	6.25	2.00	0.25	N.	Still shows desquamation on hands and feet.
June 17, 8 A. M.	26	98.1	5,800	73.25	11.75	13.25	1.75	0	N.	
June 20, 9:30 A. M.	29	7,700	65.00	21.75	10.50	2.75	0	N.	Discharged well June 20.

CASE XI.

DIAGNOSIS — MEASLES.

Female, age twenty-two years. Past history: "Measles at the age of one week;" typhoid ten years ago.

Present illness began June 11, with headache, sore throat, followed by cough, lachrymation, and sneezing, and discharge from the right ear. Rash appeared on June 13, first on the face.

Physical examination, June 13: Well-developed, stout woman. Nasal and aural discharge; moderate conjunctivitis; throat red; Koplik spots on both cheeks; eruption on hard palate; on the skin a maculopapular eruption, confluent on the face, discrete on the trunk and thighs, none on the legs. Heart not enlarged; slight systolic murmur; occasional rales.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
June 13, 5 P. M.	3	99.2	2,500	81.75	14.25	3.50	0.50	0	—	Hg. 80%; on 14th temperature 104; rash out on legs.
June 15, 3 P. M.	5	101.2	3,200	72.40	24.60	3.00	0	0	—	Brilliant rash.
June 17, 12 M.	7	98.0	4,000	51.50	36.00	10.50	2.00	0	—	Rash fading on 16th; abundant desquamation; conjunctivitis.
June 20, 7:30 A. M.	10	98.0	5,400	53.50	36.00	8.75	1.75	0	N.	No discharge from ear; conjunctivitis better.
June 24, 9:30 A. M.	14	98.5	11,300	75.00	17.00	7.50	0.50	0	N.	Up on 22d.
June 28, 2:30 P. M.	18	10,100	66.00	26.00	6.75	0.75	0.50	N.	Discharged well July 4; still soft systolic murmur at apex.
July 2,	22	19,300	56.00	39.25	3.50	1.00	0.25	—	

CASE XII.

DIAGNOSIS — MEASLES.

Female, Syrian, age four and one-half years. Sister of Case XXI. Present illness began June 11.

Physical examination, June 13: Fairly developed and nourished. Considerable prostration; conjunctivitis; profuse nasal discharge, and tender glands in the neck; many Koplik spots; eruption on the roof of the mouth and on the body, where it is sparse, consisting of scattered single macules and papules on face, trunk, arms, and thighs. Slight systolic murmur at apex; fine moist râles.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
June 13, 6:30 P.M.	4	102.0	8,400	65.75	28.50	5.50	0	0.25	N.	Hg. 65%; slight achromia; 2 myelocytes.
June 15, 3:30 P.M.	6	102.5	8,900	73.00	25.00	2.00	0	0	N.	Rash now typical and profuse; cough in- creased and a few râles; 5 myelocytes.
June 17, 11:30 A.M.	8	98.0	10,600	75.25	17.00	7.25	0.25	0.25	Desquamation on 16th; crisis today; rash fading; 1 myelocyte.
June 20, 10 A.M.	11	98.1	13,800	66.00	25.75	7.00	1.25	0	N.	Faded remains of rash visible; cough and a few râles; 2 myelocytes.
June 23, 9:30 A.M.	14	98.0	12,500	62.75	30.50	5.50	0.75	0.50	N.	Up.
June 27, 9:30 A.M.	18	98.5	14,700	64.50	27.75	6.50	0.75	0.50	N.	Red cells normal; Hg. 75%.
July 1, 2:30 P.M.	22	98.5	8,700	69.75	23.50	5.00	1.50	0.25	N.	Good conva- lescence.
July 5, 1 P.M.	26	12,800	71.25	22.75	5.50	0.50	0	N.	Discharged well July 10.

CASE XIII.

DIAGNOSIS — MEASLES.

Male, age 1 year, 4 months. Is said to have had measles before. Entered the hospital with scarlatina April 23, and had a fairly mild attack, complicated by otitis media and adenitis. On June 12 the temperature rose to 102°, and there were cough and restlessness: no Koplik spots. On the 14th a typical measly eruption appeared on the face, and sparsely over the body: cough, coryza, sneezing. Patient is well developed and fairly nourished: Koplik spots present. Heart and lungs negative.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
May 18, 11:30 A. M.	98.8	9,900	51.00	41.50	3.75	3.75	0	Exposed May 22.
June 14, 9 A. M. . .	3	102.1	5,600	59.25	36.25	4.50	0	0	N.	Hg. 65%; achromia of red cells.
June 17, 10 A. M. .	6	98.8	10,100	40.75	48.50	9.00	1.25	0.50	N.	Crisis on 15th; rash nearly gone; suppurating glands incised and drained; achromia and poikilocytosis of red cells
June 19, 8 A. M. . .	8	98.7	13,700	58.75	31.50	8.50	1.25	0	N.	Cytosis; 1 megaloblast; 1 myelocyte.
June 22, 10:30 A. M.	11	98.2	7,600	44.00	41.00	11.75	2.50	0.75	N.	Abscess doing well.
June 26, 11 A. M. .	15	98.5	8,000	36.25	48.75	13.50	1.25	0.25	N.	Glands healed: achromia, but no nucleated reds.
July 1, 3 P. M. . . .	20	98.4	7,800	39.00	43.50	14.50	1.50	1.50	Sl. +	Discharged well July 26.
July 5, 3:30 P. M. .	24	10,300	15.00	66.00	18.50	0.50	0	+	

CASE XIV.

DIAGNOSIS — MEASLES.

Male, age twenty-seven years. Measles, scarlatina, and influenza in childhood. On June 13 nausea, anorexia, pain in the legs. No cough, coryza, or lachrymation. Rash appeared on evening of June 14 on back of neck and shoulders.

Physical examination, June 15: Well developed and nourished. Slight redness of conjunctivæ and suffusion; fauces congested: macular eruption on hard palate and Koplik spots on the cheeks; typical maculopapular eruption all over body, most marked about shoulders. Faint systolic murmur: occasional fine moist râles.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
June 15, 2 P. M. . .	3	102.2	7,000	81.00	13.00	6.00	0	0	—	Hg. 90%.
June 17, 10:30 A. M.	5	99.7	5,600	62.00	27.75	8.75	1.25	0.25	—	Rash almost gone; euphoria; very mild case.
June 19, 7:30 A. M.	7	98.7	7,000	54.00	30.00	14.75	1.00	0.25	N.	Desquamation began yesterday; up on 20th.
June 22, 10 A. M. .	10	98.0	11,100	71.75	20.50	4.50	2.75	0.50	N.	Hg. 90%.
June 26, 11:30 A. M.	14	7,900	63.75	31.00	5.00	0	0.25	Sl. +	Discharged well June 27.
July 1, 2 P. M. . . .	19	6,000	58.50	28.25	10.75	1.75	0.75	N.	Returned for examination of blood.
July 6, 2 P. M. . . .	24	6,400	52.00	35.75	12.00	0	0.25	Sl. —	Ditto.

CASE XV.

DIAGNOSIS — MEASLES.

Female, age one year. Patient entered the hospital with scarlatina March 14, and was discharged well June 5. June 15 she re-entered the hospital with measles. History not obtained.

Physical examination, June 15: Fairly developed and nourished; general condition good. Coryza; conjunctivitis with photophobia; very numerous Koplik spots; no rash. Slight systolic murmur at apex; a few râles.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
June 15, 4 P. M. . .	3	100.4	11,900	38.25	54.50	6.75	0.25	0.25	—	Hg. 85%; rash appeared on 16th on face and chest.
June 17, 2 P. M. . .	5	102.9	5,400	60.50	34.00	5.50	0	0	—	Typical blotchy rash on face, scattered on trunk; coryza and cough.
June 19, 10 A. M. . .	7	99.2	8,900	31.50	57.25	9.50	1.75	0	N.	Rash at its height yesterday; numerous moist râles; 1 normoblast; no achromia.
June 22, 11 A. M. . .	10	99.1	22,400	44.25	44.75	9.50	1.50	0	+	Râles fewer; leucocytosis probably due to small patch pneumonia.
June 25, 6 P. M. . .	13	99.1	20,900	33.00	53.00	1.36	0.4	0	N.	Still numerous râles and cough.
June 28, 3 P. M. . .	16	98.5	16,000	36.25	55.00	8.50	0.25	0	Sl. +	Temperature 101.2° on 15th; cough and few râles; coryza.
July 2, 2:30 P. M. .	20	100.1	23,100	46.75	43.75	9.50	0	0	N.	No râles; still coughs. Discharged well July 7.
July 6, 2:30 P. M. .	24	12,500	36.00	55.00	7.00	2.00	0	—	

CASE XVI.

DIAGNOSIS — MEASLES, LARYNGITIS, OTITIS MEDIA.

Male, Italian, age twenty-five years. No history obtained.

Physical examination, May 7: Well developed, fairly nourished. Conjunctivae reddened: Koplik spots present: characteristic maculopapular eruption all over body. Many scattered râles.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
May 8, 5 P. M. . . .	6±	100.7	4,400	61.25	17.50	7.75	13.50	0	—	Hg. 90%; reds 5,024,000.
May 11, 11 A. M. . .	9	100.0	8,800	74.75	14.25	4.00	6.75	0.25	—	Very hoarse; rash nearly gone on 12th. purulent discharge from right ear.
May 14, 5 P. M. . . .	12	101.9	9,900	51.50	31.50	5.50	10.50	1.00	N.	Severe bronchitis still with hoarseness
May 18, 4 P. M. . . .	16	100.0	10,600	69.25	20.25	6.25	3.00	1.25	—	Hg. 90%; reds 4,868,000; less cough; still râles and hoarseness.
May 22, 4 P. M. . . .	20	99.0	10,400	67.50	21.00	5.25	5.75	0.50	N.	Ear still discharging; râles.
May 26, 10:30 A. M. .	24	99.0	10,200	60.25	28.50	2.00	8.00	1.25	N.	Hg. 90%; reds 5,328,000; diarrhoea; slight aural discharge.
June 1, 11:30 A. M. .	30	98.6	9,600	56.50	23.75	8.00	11.00	0.75	N.	Up on 28th.
June 7, 8 A. M. . . .	36	98.6	9,000	74.50	14.75	3.50	6.75	0.50	—	Discharged well.

CASE XVII.

DIAGNOSIS — MEASLES, OTITIS MEDIA, MASTOIDITIS, PHTHISIS.

Female, age fourteen years. Past history: Chronic discharge from both ears, off and on, since age of seven. Present illness began May 19 with headache, fever, coryza.

Physical examination, May 21: Fairly well developed and nourished. Conjunctivae and throat injected: Koplik spots present: characteristic rash on roof of mouth and skin, confluent on the face, discrete elsewhere. Heart negative; moist and dry râles present. Temperature 104.8°.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
May 22, 3:30 P.M.	4	102.0	5,900	83.75	13.00	3.25	0	0	—	Hg. 90%; red cells 5,356,000; rash same as yesterday.
May 24, 6 P. M. . . .	6	98.5	7,200	49.00	41.50	8.00	1.50	0	N.	Rash fading; desquamation beginning.
May 26,	8	98.2	9,000	56.25	34.50	7.25	1.75	0.25	+	Rash gone.
May 29, 6 P. M. . . .	11	99.3	12,500	69.75	22.00	8.25	0	0	N.	Right ear began to discharge yesterday; left ear aches.
June 1, 10:30 A.M.	14	100.2	16,200	76.75	16.75	6.50	0	0	+	Temperature 101 yesterday; left ear now discharging; partial deafness.
June 4, 5 P. M. . . .	17	103.2	14,700	83.25	13.75	2.50	0.25	0.25	—	Profuse discharge; no mastoid tenderness; 1 myelocyte.
June 7, 6 P. M. . . .	20	101.6	9,200	62.00	31.50	5.25	1.25	0	—	Less discharge.
June 12, 3:30 P.M.	25	99.9	11,300	73.00	21.00	4.50	1.50	0	N.	No discharge from left ear, slight from right.
June 16, 9:30 A.M.	29	98.7	16,100	70.75	24.50	3.75	0.50	0.50	++	Tender swelling in front right ear appeared yesterday; right ear still running.
June 20, 10:30 A.M.	33	98.3	8,700	59.50	33.00	7.50	0	0	+	
June 23, 9 A. M. . .	36	98.0	9,800	77.50	16.25	6.00	0.25	0	Sl. —	Swelling in front of ear persists; edema of anterior wall of auditory canal; swelling and tenderness over mastoid.
June 26, 9:30 A.M.	39	100.0	7,100	66.50	21.50	11.75	0	0.25	Sl. —	On 25th slight hemoptosis, temperature 103; no tubercle bacilli in sputum; scattered coarse râles; cervical glands enlarged. On 26th operation: mastoid full of pus; curetted and drained.
June 29, 10:30 A.M.	42	100.2	10,900	47.75	41.50	10.25	0.25	0.25	N.	Moderate fever till July 21; July 29 signs of infiltration at left apex. Discharged to Convalescent Home.

CASE XVIII.

DIAGNOSIS — MEASLES, OTITIS MEDIA, MASTOIDITIS.

Female, age twenty-three years. Acute articular rheumatism five years ago. Onset of present illness May 23, with cough and rash, appearing first on forehead.

Physical examination. May 27: Well developed and nourished. Eyes suffused; mouth and throat reddened; macular eruption on hard palate; Koplik spots present: on the skin there is a bright maculopapular rash, confluent on the face, elsewhere discrete. The heart shows enlargement to the left, a systolic murmur at the apex, and accented pulmonic second sound (mitral regurgitation); lungs negative.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
May 27, 5 P. M. . .	5	104.0	4,800	84.50	11.50	4.00	0	0	+	Strych. gr. $\frac{1}{40}$ every 4 hours.
May 29, 5 P. M. . .	7	100.8	4,700	61.50	20.00	10.00	7.75	0.75	N.	Rash fading.
May 31, 1 P. M. . .	9	97.7	4,300	49.00	37.00	10.25	3.50	0.25	N.	Cough; lungs negative; rash not quite gone.
June 2, 6 P. M. . .	11	98.6	16,300	79.75	13.75	4.00	1.75	0.75	N.	Yesterday ear-ache on right with congestion and bulging of drum head.
June 3, 3:30 P.M.	14	99.2	10,000	68.75	20.00	8.50	1.75	1.00	N.	Right ear still aches, but does not discharge; desquamation.
June 8, 10 A. M. .	17	100.0	14,700	74.00	16.25	8.25	1.00	0.50	N.	Purulent discharge from right ear since yesterday; irrigation.
June 12, 4 P. M. .	21	100.4	13,100	68.50	25.00	3.50	1.00	2.00	N.	Still much aural discharge.
June 16, 9 A. M. .	25	99.5	10,800	74.25	11.50	10.25	2.00	2.00	Sl.+	Gaining strength slowly; paracentesis of right ear on 21st.
June 20, 10 A. M.	29	99.0	12,900	63.75	23.50	7.75	2.75	2.25	+	Discharge continues; tender over mastoid 2 weeks; July 8 operation and pus found. July 29 discharged relieved.
July 7, 6:30 P.M.	46	98.7	11,300	64.00	26.00	5.50	2.75	1.75	...	

CASE XIX.

DIAGNOSIS — MEASLES, TONSILLITIS.

Male, age twenty-three years. Past history negative. Present illness began May 18 with cough, fever, and lachrymation.

Physical examination, May 23: Well developed and nourished. Eyes suffused; throat red: Koplik spots present; typical maculopapular eruption on face and body, thickest on face. Heart and lungs negative.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
May 23, 6 P. M.	6	102.3	8,900	85.25	9.75	4.25	0.50	0.25	—	
May 25, 5:30 P. M.	8	98.3	10,700	56.50	36.25	5.75	1.50	0	N.	Rash fading.
May 29, 3:30 P. M.	12	98.2	14,000	73.25	20.00	5.75	0.75	0.25	N.	Few sonorous rales; less cough; desquamation yesterday.
June 2, 6:30 P. M.	16	98.0	19,000	72.00	19.50	7.25	0.75	0.50	N.	May 31, sore throat; fever 102.8; tonsils swollen with exudate.
June 4, 6:30 P. M.	18	99.0	19,000	72.00	23.50	3.75	0	0.75	N.	Exudate still present on tonsils; up since 3d.
June 5, 9 A. M.	20	17,800	74.50	20.25	4.50	0.50	0.25	N.	
June 10, 10:30 A. M.	24	98.5	16,600	67.50	25.25	6.50	0.25	0.50	N.	Chronic pharyngitis and enlarged tonsils; no exudate; June 12, discharged well.

CASE XX.

DIAGNOSIS—MEASLES DURING CONVALESCENCE FROM SCARLATINA, DIPHTHERIA.

Male, age nine years. Past history: Scarlatina beginning April 29: on May 14 fever to 102.7°: next day cough, sneezing: Koplik spots. Glands in neck much enlarged: no rash.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
May 15, 5 P. M. . .	2	101.1	16,700	74.80	9.80	13.21	1.20	0.80	N.	Hg. 75%; reds 4,180,000; leucocytosis may be due to previous scarlatina.
May 17, 5:30 P. M.	4	100.0	8,300	76.25	11.75	11.75	0	0.25	Sl. +	More Koplik's spots; euphoria; suffusion of eyes.
May 18, 5 P. M. . .	5	100.0	9,500	83.75	6.75	9.25	0	0.25	N.	Ether at 12 M. for extraction of tooth.
May 19, 3:30 P. M.	6	103.4	7,500	79.75	8.00	11.25	0	1.00	—	Typical rash appeared first on face then on whole body except abdomen; coryza; cough.
May 20, 5:30 P. M.	7	103.4	7,300	74.00	18.50	7.00	0.50	0	N.	Very profuse rash.
May 21, 3:30 P. M.	8	101.2	11,800	73.25	18.75	7.00	1.00	0	N.	Rash fading; desquamation of scarlatina on thigh; numerous dry rales.
May 23, 5 P. M. . .	10	102.8	32,000	76.00	19.25	4.25	0.25	0.25	—	Stenosis larynx intubation, antitoxine 12,000 units; coughed up membranes; 1 myelocyte seen.
May 24, 5 P. M. . .	11	103.0	24,100	71.50	21.75	6.75	0	0	N.	Breathes easily through tube; Hg. 80%; red cells 5,000,000.
May 26, 12:30 P. M.	13	102.0	21,900	66.00	22.50	11.50	0	0	Much +	Positive culture from nose and throat; extubed yesterday; strychn. $\frac{1}{80}$ and brandy every 4 hours.
May 30, 6 P. M. . .	17	99.2	17,800	78.00	14.75	6.25	0.50	0.50	+	Purulent discharge from ears, and culture shows diphtheria bacilli.
June 4, 4:30 P. M.	22	98.6	16,600	79.50	15.75	4.50	0.25	0	—	Aphonia; achromia in red cells.
June 11, 9:30 A. M.	29	98.4	11,900	69.75	18.75	11.00	0.25	0.25	N.	Reds 4,696,000; Hg. 55%; convalescent. Discharged well July 4.

CASE XXI.

DIAGNOSIS — MEASLES, BRONCHOPNEUMONIA, DIPHTHERIA.

Male, Syrian, age two years. One sister has measles (case No. 12). Present illness began June 10.

Physical examination, June 13: Fairly nourished, marked rachitis: throat red: a slight eruption on hard palate: Koplik spots on both cheeks: on face, neck, and chest a fine discrete maculopapular rash, less marked on the abdomen, thighs, and arms; cervical glands enlarged. Systolic murmur at apex; marked general bronchitis: spleen palpable two fingers below costal margin.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
June 13, 6 P. M.,	4	99.0	8,100	64.50	25.00	10.00	0	0.50	—	Hg. 85%; strychn. gr. $\frac{1}{100}$ every 4 hrs.
June 15, 2:30 P. M.	6	102.7	9,100	50.50	37.25	11.75	0.25	0.25	—	Eruption sparse until yesterday, now at its height; nasal discharge; dry râles; 2 myelocytes.
June 17, 11 A. M.,	8	100.3	13,100	52.50	35.25	11.25	1.00	0	—	Rash fading; desquamation; 1 myelocyte.
June 20, 8 A. M.,	11	99.0	19,100	60.25	28.75	9.25	1.00	0.75	—	Rash still visible till today; râles more numerous and moist; expiratory grunt; 2 myelocytes.
June 23, 9 A. M.,	14	99.5	15,900	62.50	30.25	6.00	0.50	0.75	+	On 21st temperature 102.3; bronchial breathing and consonating râles at right apex; no signs of consolidation; 2 myelocytes.
June 26, 10 A. M.,	17	99.7	15,300	64.75	28.25	6.00	0.75	0.25	N.	Few râles; laryngeal cough; slight difficulty in breathing.
June 28, 10:30 A. M.	19	101.0	31,800	84.25	12.00	3.75	0	0	N.	Stenosis larynx; intubation with relief; antitoxin 12,000 units.
July 1, 1:30 P. M.	22	99.8	20,050	77.25	15.50	6.75	0.25	0.25	N.	On 29th positive culture; coughed up pieces of membrane; antitoxin 12,000 units.
July 5, 12:30 P. M.	26	101.3	15,800	72.25	17.25	8.25	2.25	0	N.	July 8 died in fit of suffocation; autopsy showed ulcer in larynx, probably due to badly fitting tube.

CASE XXII.

DIAGNOSIS — MEASLES, DIPHTHERIA.

Male, age about twenty-five years. No history obtained, except that the rash appeared May 25.

Physical examination, May 26: Well developed and nourished. Marked conjunctivitis with photophobia; tonsils swollen and red, with mucus exudate; throat very red; macular rash on roof of mouth; numerous Kopliks: typical maculopapular rash on face and to a slight degree on the chest, not elsewhere. Heart negative; no râles.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
May 26, 4 P. M. . .	5 ±	101.5	4,400	81.00	12.75	5.00	1.25	0	—	Hg. 95; red, 6,420,000.
May 29, 2:30 P. M.	8	103.8	5,000	89.75	7.00	3.00	0.25	0	—	Brilliant universal rash; severe conjunctivitis; enlarged and tender glands under jaw.
May 31, 12 M. . . .	10	100.5	8,100	62.25	24.50	9.25	4.00	0	—	Rash fading; petechæ in places; numerous dry râles; conjunctivitis better.
June 2, 5 P. M. . .	12	101.0	10,000	78.75	15.50	4.00	1.75	0	N.	Desquamation; throat red and inflamed; tonsils swollen, but without exudate.
June 5, 9 A. M. . .	15	100.3	15,700	85.00	8.75	6.25	0	0	N.	On 6th sore throat, hoarseness; temperature 101.
June 7, 8:30 A. M.	17	99.0	14,200	87.75	7.75	4.00	0.50	0	N.	Very hoarse; no râles; tonsils red swollen, with thick exudate at mouth of crypts.
June 9, 9:30 A. M.	19	98.8	19,300	93.75	3.50	2.50	0.25	0	—	On 8th positive culture from throat; antitoxin 8,000 units on 7th and 8th.
June 12, 8 A. M. . .	22	99.3	10,300	90.50	3.75	3.75	1.25	0.75	+	Upon 14th; slight chronic pharyngitis; discharged well June 25.
June 17, 9 A. M. . .	27	98.5	9,700	73.50	19.50	5.25	1.25	0.50	+	

CASE XXIII.

DIAGNOSIS — MEASLES, DIPHTHERIA.

Female, age ten years. Pneumonia four years ago; no children's diseases. Present illness began June 10 with malaise, nausea, and headache; later, cough and sore eyes. Rash appeared on evening of June 14, on face and then on chest.

Physical examination, June 15: Well developed and nourished. Moderate prostration; slight conjunctivitis and coryza; a few Koplik spots on each cheek; characteristic early eruption of measles; thick on the face, thinner elsewhere. Heart negative; a few fine râles.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocyte %	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
June 15, 1 P. M. . .	5	101.0	4,400	83.25	12.25	3.50	0	1	—	Hg. 80; crisis on 16th; mild case.
June 17, 8:30 A. M. .	7	98.3	4,700	55.00	30.25	13.75	0.75	0.25	—	Rash fading; euphoria.
June 19, 9:30 A. M. .	9	98.2	6,000	51.00	36.50	9.25	1.75	1.50	N.	Slight desquamation.
June 21, 12:30 P. M. .	11	101.6	17,900	78.75	15.00	6.25	0	0	—	Sore throat; gray membrane on right tonsil, little on left; positive culture; antitoxin 8,000 units.
June 22, 3:30 P. M. .	12	100.0	21,400	82.75	8.75	8.00	0.50	0	+	Comfortable.
June 25, 7 P. M. . .	15	98.5	14,500	59.25	26.50	8.50	3.75	2.00	..	Membrane rapidly disappeared.
June 28, 12:30 P. M. .	18	100.3	19,900	80.50	14.25	3.50	1.75	0	N.	Urticaria; fever from antitoxin; increased leucocytosis and rise in neutrophils, probably from same cause.
July 2, 5 P. M. . . .	22	99.0	7,200	48.50	38.25	11.25	2.00	0	Sl.—	Digestive leucocytosis; discharged well July 8.
July 5, 3 P. M. . . .	25	98.5	13,200	64.60	27.60	6.00	1.00	0.80	+	

CASE XXIV.

DIAGNOSIS — MEASLES, DIPHTHERIA

Male, age twenty-three years. Scarletina, pertussis, and mumps in childhood. Malaria eleven years ago. Present disease began June 7 with fever, cough, hoarseness, and coryza. Two days later a rash appeared, first on the face, then on the body, with soreness of the eyes.

Physical examination, June 10: Well developed and nourished. Slight conjunctivitis; numerous Koplik spots; eruption on roof of mouth; characteristic blotchy eruption of measles on face and body. Soft systolic murmur at apex; scattered moist râles.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
June 10, 10:30 A.M.	4	104.2	5,100	84.75	6.75	8.25	0	0.25	—	Hg. 90%; urine—no albumin; cultures from nose and throat negative.
June 12, 8:30 A.M.	6	98.7	4,900	60.50	23.50	11.00	4.75	0.25	—	Brilliant eruption, confluent on face and mouth; negative culture.
June 14, 8:00 A.M.	8	98.8	12,600	74.00	21.00	4.50	0.50	0	—	Eruption gone except where it was petechial; voice hoarse.
June 17, 9:30 A.M.	11	98.2	21,000	83.25	6.00	10.25	0.25	0.25	+	Still hoarse; throat, heart, lungs, and abdomen negative.
June 19, 8:30 A.M.	13	99.0	29,800	85.25	7.75	6.75	0	0.25	N.	Chill yesterday with rise of temperature to 103.3; nothing objective.
June 22, 12 M.	16	100.0	18,100	—	Does not appear sick.
June 25, 5:30 P.M.	19	99.0	15,600	Hoarseness wearing off; negative culture from nose and throat.
June 30, 3 P.M.	24	99.0	19,100	83.75	11.25	4.25	0.50	0.25	+	Discharged well June 30.

CASE XXV.

DIAGNOSIS—MEASLES DURING CONVALESCENCE FROM TYPHOID FEVER.

Male age sixteen years. Entered the Massachusetts General Hospital May 5, 1902, giving a history of two weeks of symptoms suggesting typhoid fever. On May 17, during convalescence from the fever, the temperature was accompanied by slight general symptoms, and on the third day a characteristic rash appeared.

Physical examination, May 20: Well-developed, moderately emaciated boy. Eyes suffused; numerous Koplik spots present; marked macular eruption on soft palate; typical eruption of macules and papules on face, trunk, and thighs, sparse on the legs, confluent on the back. Systolic murmur at apex; lungs negative; spleen not palpable, but enlarged to percussion.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
May 20, 4:30 P.M.	6 ±	101.5	3,400	65.25	29.50	3.50	1.75	0	N.	Red cells 4,336,000; Hg. 65%.
May 22, 4:30 P.M.	8	98.1	5,100	47.00	46.50	4.75	1.75	0	—	Temperature fell yesterday; rash nearly gone; desquamation beginning.
May, 24.....	10	98.3	4,700	51.00	43.25	4.50	1.25	0	—	Convalescent.
May 27, 6 P.M....	13	97.8	5,500	62.00	30.50	5.75	1.50	0.25	N.	Red cells show slight achromia Up on 5th.
May 31, 2 P.M....	17	98.5	5,200	60.75	33.00	5.25	0.75	0.25	Sl.—	
June 6, 7 P.M....	23	100.5	3,700	59.75	34.25	5.25	0.25	0.50	—	Good convales- cence. Discharged well July 2.
June 11, 8:30 A.M.	28	97.7	2,900	43.75	52.00	2.00	1.50	0.25	—	
June 18, 8 A.M...	35	98.5	4,500	58.50	31.50	8.25	1.25	0.50	—	
June 26, 10:30 A.M.	43	5,100	55.75	36.25	6.75	0.75	0.50	—	

CASE XXVI.

DIAGNOSIS — SCARLATINA, MEASLES.

Female, age three years. No history obtained.

Physical examination, May 31: Fairly developed and nourished. Slight nasal discharge; mouth and throat red; papillae of tongue red and swollen; punctate eruption on roof of mouth and over chest and abdomen, emphasized in the groins and axillae, typical of scarlatina. Heart and lungs negative.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
May 31, 4 P. M.	2	101.8	9,300	67.75	21.25	7.75	3.00	0.25	+	Hg. 85%.
June 2, 4:30 P. M.	4	104.2	7,000	68.75	20.75	8.75	1.50	0.25	Characteristic maculopapular rash all over body; Koplik spots; 7 myelocytes.
June 4, 4 P. M.	6	99.3	12,300	58.50	34.00	4.25	3.25	0	—	Crisis; rash almost gone; no desquamation; conjunctivitis; 3 myelocytes; moderate achromia.
June 6, 6:30 P. M.	8	98.1	19,600	58.25	33.75	5.00	2.75	0.25	+	Still much cough; 11 myelocytes.
June 8, 11:30 A. M.	10	98.4	19,200	48.25	41.25	7.50	2.75	0.25	N.	A few coarse rales, otherwise nothing objective; red cells normal; no myelocytes.
June 12, 3 P. M.	14	98.7	22,600	62.75	24.50	9.25	3.00	0.50	1 myelocyte.
June 16, 10:30 A. M.	18	98.7	16,600	57.00	33.75	5.50	3.00	0.75	N.	Typical desquamation of scarlatina.
June 21, 12 M.	23	98.4	13,600	39.25	49.50	7.75	1.75	1.75	Sl.—	Up yesterday; 1 myelocyte.
June 25, 6:30 P. M.	27	18,900	44.25	46.50	7.50	1.25	0.50	N.	Still desquamating.
June 29, 10 A. M.	31	14,400	52.50	34.00	9.50	3.75	0.25	N.	Discharged well July 21.
July 3, 2:30 P. M.	35	13,800	43.75	45.75	9.00	1.75	0.75	

CASE XXVII.

DIAGNOSIS — SCARLATINA, MEASLES, OTITIS MEDIA.

Male, age seven years. Transferred from Eye and Ear Infirmary, where he had undergone an operation for adenoids. On May 29 he was seized with fever and malaise, and the next day a punctate erythematous eruption was noted. Entered the hospital May 31.

Physical examination, May 31: Well developed and nourished. Facies of adenoids; discharge from left ear: marked conjunctivitis and blepharitis: punctate eruption on roof of mouth: papillae of tongue, red and enlarged: throat red; over body a punctate erythema, fading on chest, bright on extremities. Heart and lungs negative.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
May 31, 3:30 P. M.	0	102.8	6,900	73.50	11.00	5.00	10.25	0.25	N.	Hg. 85%.
June 2, 3:30 P. M.	2	102	7,000	68.25	16.75	10.25	4.25	0.50	Rash fainter; eyes suffused; Koplik spots.
June 4, 6 P. M. . .	4	101.2	4,600	79.75	14.75	3.50	1.75	0.25	—	Typical maculopapular eruption on face, body, extremities, and hard palate; Koplik spots; cough.
June 5, 5 P. M. . .	5	100.1	4,500	71.75	23.00	3.25	1.25	0.75	N.	
June 7, 6:30 P. M.	7	100.4	4,600	60.25	24.25	10.25	5.25	0	—	On 6th rash profuse, confluent on body; temperature 103.2°.
June 9	9	100.3	9,100	72.25	20.75	5.00	1.75	0.25	—	Earache; no discharge; drum opaque.
June 12, 9 A. M. . .	12	99.9	11,700	69.25	16.75	7.25	6.25	0.50	N.	No symptoms; some achromia.
June 16, 10 A. M.	16	100.1	8,400	69.00	20.50	8.00	1.50	1.00	N.	Slight purulent discharge from both ears; partially deaf; chronic rhinitis and blepharitis.
June 21, 11 A. M.	21	98.4	10,300	61.75	24.75	9.25	2.50	1.75	—	Hg. 55%; red cells 4,248,000; reds show achromia.
June 29, 9 A. M. .	27	7,800	48.50	37.00	11.00	3.25	0.25	+	Ear still discharging, August 1, discharged.

CASE XXVIII.

DIAGNOSIS — MEASLES, SCARLATINA DURING CONVALESCENCE.

Male, age five years. Past history negative. Present illness began June 10 with coryza: and rash appearing on the face.

Physical examination, June 10: Well developed and nourished. Suffusion of eyes: coryza: conspicuous Koplik spots; on the face is a discrete maculopapular eruption: a few spots on the chest, none elsewhere. Heart and lungs negative.

Date	Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells	Blood Plates	Remarks
June 10, 7 P. M.	4	103.1	7,800	76.00	18.25	4.75	0.25	0.75	N.	Hg. 80%; crisis on 12th.
June 12, 2 P. M.	6	99.5	5,800	49.75	33.75	12.50	4.00	0	—	Eruption, fully out yesterday, still bright to-day; condition good.
June 14, 8:30 A. M.	8	98.4	6,700	38.50	50.50	7.00	3.75	0.25	N.	Eruption fading away; occasional moist rale.
June 17, 10:30 A. M.	11	98.7	9,800	65.00	25.00	5.75	3.50	0.75	N.	Desquamation on 16th.
June 20, 8:30 A. M.	14	99.2	11,400	72.25	21.25	5.00	1.50	0	N.	Ear cold and cyanotic when blood taken; this might account for leucocytosis.
June 23, 8:30 A. M.	17	99.3	17,000	73.50	18.25	5.50	2.50	0.25	N.	
June 26, 9 A. M.	20	99.5	6,700	64.25	21.00	9.75	4.25	0.75	N.	No symptoms.
June 28, 4 P. M.	22	104.0	21,600	79.25	15.50	3.50	1.75	0	—	Sore throat, fever, malaise; throat red, erythematous rash on neck and chest; scarlatina.
June 30, 7:30 A. M.	24	99.2	25,600	89.75	4.75	4.00	1.25	0.25	—	Typical punctal erythema on body and roof of mouth yesterday; Hg. 65%; reds 4,644,000.
July 2, 3 P. M.	26	101.0	18,600	66.25	16.50	10.50	6.75	0	N.	Rash fading, but still present; desquamation.
July 5, 4 P. M.	29	99.8	19,300	59.75	29.00	6.25	4.50	0.50	N.	Euphoria. Discharged well August 20.

The subjoined table, based on fourteen cases in adults, will give an idea of the course of the white and differential counts, at different periods of the disease. It is, of course, not mathematically exact, being based on too few cases, for as the blood was not examined every day, some of the averages are made up from only

a few cases. It is obvious that cases running clinically a more rapid course will pass through the usual blood changes in a shorter time.

Day of Disease	Temperature	White Corpuscles	Polynuclear Leucocytes	Small Mononuclear Leucocytes	Large Mononuclear Leucocytes	Eosinophiles	Mast Cells
3	100.7	4,700	81.4	13.6	4.7	0.25	0
4	102.4	5,900	81.0	13.3	5.4	0.1	0.2
5	101.6	4,800	74.4	18.5	5.7	0.6	0.2
6	100.4	6,800	68.9	20.9	8.2	1.7	0.2
7	99.1	6,400	55.5	29.9	10.5	3.9	0.2
8	98.4	10,800	62.2	30.6	5.8	1.0	0.1
9	98.5	5,800	53.2	35.4	9.9	2.9	0.6
10	98.6	10,200	64.5	25.0	7.1	2.9	0.3
12	98.2	9,800	70.6	21.2	6.6	1.4	0.3
14	98.4	8,800	66.9	25.8	7.8	1.2	0.2

The following cases are of interest as showing the effect of complications:

Case XXIX. Diagnosis—measles, bronchopneumonia. Boy, age two years. Eighth day of disease. Rash appeared four days ago. Dyspnea, general bronchitis, well-marked consolidation at left upper lobe. Temperature, 100.8°. White count, 15,800. Differential count: neutrophiles, 57.50 per cent.; small lymphocytes, 31.50 per cent.; large lymphocytes, 5 per cent.; eosinophiles, 3.25 per cent.; mast cells, 0.25. 10 myelocytes. 1 megaloblast. Death on following day.

Case XXX. Diagnosis—measles, osteomyelitis. Girl, age eight years. Sixth day of disease. Fading rash. White count, 21,300. Neutrophiles, 80 per cent.; count rose to 42,700 later. Suppurating focus found at operation in upper epiphysis of tibia.

Case XXXI. Diagnosis—measles, parotitis epidemica. Boy, age eight years. Ninth day of disease. Rash nearly gone. Both parotids swollen and painful. Temperature, 100.3°. White count, 16,800. Neutrophiles, 79.50 per cent.; small mononuclears, 9.75 per cent.; large mononuclears, 6.50 per cent.; eosinophiles, 4.25 per cent. Mast cells, 0.

Case XXXII. Diagnosis—measles, mastoiditis. Boy, age six years. Twenty-seventh day of measles, ninth day of mastoid disease. White count, 14,400. Operation the same day showed a small amount of pus in the mastoid.

Case XXXIII. Diagnosis—measles, severe conjunctivitis. Girl, age five years. Seventh day of disease. Eruption at its height. White count, 9,300. Neutrophiles, 87 per cent.

Let us first consider the white count. Unfortunately no cases could be examined during the period of incubation. In the latter part of the stage of invasion the count was normal, with the excep-

tion of Case XX, where a count of 16,700 was found on the day after the first symptoms appeared. The previous scarlatina renders this count of no value, as it is known that the leucocytosis of scarlatina may continue for weeks. The count fell with the breaking out of the rash, usually reaching its lowest point at the height of the rash. At this time it was usually a low normal; in one-third of the cases it was diminished below 5,000 (leucopenia); in a few cases it was normal. With the fall of temperature the count became normal, either immediately or within a few days, sometimes rising to slightly above normal. The extent of the diminution was in general not dependent upon the severity of the disease, except that the *very* low counts appeared only in severe cases. The average minimum count was 5,900, the lowest 2,500.

The neutrophiles followed a perfectly regular course; normal or somewhat increased during the period of invasion, they showed a well-marked relative increase in the stage of eruption, followed by a marked and sudden fall with the paling of the rash and the fall in temperature, reaching subnormal values. They then gradually increased to normal during convalescence. Neutrophilic myelocytes were found in ten cases, usually most abundant at the time of the crisis. They were more frequently found in children and always in small numbers, up to 1.25 per cent. The small lymphocytes followed a course opposite to that of the neutrophiles, being normal or already decreased in the period of invasion, markedly diminished during the eruption, rising to above normal at defervescence, and regaining normal values later. The large mononuclears (including large lymphocytes and transitional forms) were normal or even increased during the stage of eruption, and rose to above normal at defervescence. The eosins began to be diminished during the invasion, and at the height of the eruption were decreased or absent altogether. At defervescence they reached high normal values or were distinctly increased; in fact, they were in every case increased above the percentage normal for that individual. The highest percentage was 13.50 per cent. (Case XVI); two other cases (X and XVIII) showed 8 per cent.

The mast cells showed no characteristic changes. The blood

plates were usually markedly decreased at the height of the rash, but in several cases they were normal, and in one increased.

In the case of children there was likewise a normal or diminished white count during the eruptive stage, and the differential count underwent changes similar to that in the adult, if we reckon from the percentages normal for the age in question. As was to be expected, the neutrophiles were diminished and the lymphocytes and large mononuclears were increased as compared with measles in the adult. An infant, for example, showed during the eruptive stage an increase of neutrophiles up to 50 per cent.

The red count was normal during the acute stage, unless lowered by previous disease. In two cases in which several counts were made there was a moderate loss in red cells after the crisis, about 200,000. The hemaglobin showed no constant changes, but the method was not sufficiently accurate to detect slight variations.

COMPLICATIONS.

Acute otitis media occurred in four cases (XVI, XVII, XVIII, and XXVII), in three it caused leucocytosis, in all an increase in the neutrophiles. In two of these (XVII and XVIII) mastoiditis occurred later, and at operation the white and differential counts were practically normal, although pus was found. This shows us that in mastoiditis, as in appendicitis, the absence of leucocytosis rules out only a suppurating process which is in the acute stage. A third case of mastoiditis (XXXII) had a count of 14,400 on the ninth day; pus was found at operation. Bronchopneumonia in two cases (XXI and XXIX) was accompanied by leucocytosis. It probably accounted for the leucocytosis in Case XV, in which only the signs of extensive bronchitis could be detected. Scarlatina occurring simultaneously with measles in two cases (XXVI and XXVII) showed a normal white count, but increased eosins. This is interesting as showing how the two diseases interacted upon each other, measles abolishing the leucocytosis normal to scarlatina, and the latter increasing the eosins over the normal for measles. During convalescence the scarlatinal leucocytosis appeared in one case, but not in the other. Scarlatina complicating convalescence from measles (Case XXVIII) produced leuco-

cytosis with increased neutrophiles and eosins, as is usual in that disease. The complication with diphtheria (Cases XX-XXIII) always gave a well-marked leucocytosis. In two cases a rise in the neutrophiles or a failure to fall at the proper time preceded the rise in the white count, and came several days before the first symptoms of the disease. Measles during convalescence from typhoid showed a marked and long-continued leucopenia and increased lymphocytes; this was to be expected from the conjunction of the two diseases producing the same effects on the blood. Tonsillitis, parotitis epidemica, osteomyelitis, and subcutaneous abscesses (Cases XIX, XXXI, XXX, and V, respectively) were all accompanied by leucocytosis. Finally it must be mentioned that in four cases (II, VII, IX, and XII) there was a moderate leucocytosis (15,000) during convalescence, for which no cause could be found. This does not militate against the value of blood examinations; it merely shows that leucocytosis may sometimes arise from causes which our present clinical methods are not sufficiently delicate to detect.

It will be noticed that Sobotka, Renaud, and Plantenga have all found a leucocytosis during the incubation period of measles. This is of considerable theoretical interest, for it shows that during the time there is a reaction on the part of the organism toward the specific virus. It should also be of value in prophylaxis in hospitals, where frequent blood counts are possible. During the stage of eruption all observers have found either normal white counts or leucopenia. This puts measles in the same class with those acute infectious diseases which run their course without leucocytosis, typhoid, influenza, and miliary tuberculosis. The cause of leucopenia is to be sought either in the production of some substance exerting a negative chemotaxis on the leucocytes, so that they are sent out from the blood-making organs in diminished numbers, or in the actual destruction of leucocytes. The writer follows Türk in adopting the former view, as more in accord with the latest work on the chemistry of the blood. Diseases which of themselves produce leucocytosis do so also when complicating measles, as a rule, but during the stage of eruption the two diseases may counteract each other, so that a

normal count results. This occurred in my cases complicated with scarlatina. The presence, therefore, of leucocytosis points to a complication; its absence does not exclude one. It is worthy of note that the catarrhal processes belonging to measles do not, even if severe, cause leucocytosis, as shown by my cases of severe bronchitis, laryngitis, and conjunctivitis (Cases XVI and XXXIII). This is of diagnostic importance in distinguishing them from bronchopneumonia and diphtheria.

The studies of the course of the differential count made by Türk (three cases), Reckzeh (ten cases), and the writer (twenty-eight cases) agree in all essential particulars. The increase in neutrophiles with decreased eosins is not peculiar to measles, but occurs in practically all acute infectious diseases and in suppuration. Scarlatina alone shows an increase of both neutrophiles and eosins during the acute stage. The later increase of eosins in measles sometimes reaches the grade of that in scarlatina, but it is usually not so great and of much shorter duration. In those cases where the rash is of doubtful character, and it is hard to decide between measles and scarlatina, the blood may help us. Scarlatina is usually accompanied by a well-marked leucocytosis. Mild cases may have a normal count, but here the eosins are usually increased; in measles, on the contrary, they are diminished. The chief practical value of blood examinations in measles lies, in my opinion, in the early detection of complications, and here a rise in neutrophiles, even without leucocytosis, is suggestive. It is not often that the diagnosis is in doubt when the rash is already out.

CONCLUSIONS.

From my own work and that of others I would draw the following conclusions:

1. During the incubation period of measles a leucocytosis is present, which begins soon after infection, reaches its maximum six days before the appearance of the eruption, and lasts into the first part of the stage of invasion.

2. During the latter part of the stage of invasion and during the stage of eruption the white count is normal or more usually diminished, reaching its lowest point at the height of the rash.

3. The presence of leucocytosis points to the existence of a complication; its absence during the eruptive stage does not exclude one. The onset of leucocytosis with a complication may be preceded by a rise in the percentage of neutrophiles, or a failure on their part to decrease at the proper time.

4. The differential count in measles takes the following definite course: the polynuclear neutrophiles are increased during the stage of incubation, invasion, and eruption, fall below normal at defervescence, and reach normal in convalescence. Myelocytes are often present in small numbers during the period of eruption, especially in young children. The lymphocytes follow the opposite course—at first decreased, and increased after the crisis. The large mononuclear and transitional cells (including large lymphocytes) are normal at first, and increased later. Eosins are decreased or absent during the eruption, and later increased. The mast cells show no changes; the blood plates are usually decreased during the eruption. The blood becomes normal in all respects at about the twelfth day.

5. In doubtful cases the absence of leucocytosis with decreased eosins is in favor of the diagnosis of measles as against scarlatina, and *vice versa*.

6. The blood of measles in children shows the same changes as in adults, if the differences between the normal blood of children and that of adults be taken into consideration.

In conclusion I wish to express my thanks to Dr. R. C. Cabot, Dr. E. A. Locke, and Dr. D. L. Edsall for valuable aid and suggestions.

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THE REDUCTION OF METHYLENE BLUE BY NERVOUS TISSUE.*

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A NUMBER of years ago Ehrlich pointed out the ability of living cells to decolorize (*i. e.*, reduce) solutions of methylene blue. Later, Neisser and Wechsberg† devised an application of this phenomenon to determine the cytocidal effect of some cellular poisons upon leucocytes. They found that living leucocytes were able to reduce the dye quantitatively, but when they were killed by heat, alcohol, an immune leucotoxic serum, or staphylococcus "leucocidin," they lost this power. Other cells, when fresh (spermatozoa, pancreas, kidney), also reduced the dye, but not after treatment with an injurious agent (alcohol). Living micro-organisms had a similar effect, which was lost on treating them with a bactericidal substance. Ferments and bacterial toxins had no reducing power.

I attempted to use this method in determining the toxic effect of an immune neurotoxic serum upon nervous tissue. The serum was prepared by immunizing rabbits and geese with the central nervous system of guinea pigs, and was generally of such strength that 2.0 c.c. intravenously, 0.5–1 c.c. in the carotid, or 0.01–0.5 c.c. intracerebrally caused the death of a guinea pig in two to twelve hours. The histological examination of tissues as reported by Ricketts and Rothstein‡ shows that this serum produces, *intra vitam*, profound changes in the nerve cells, while the effects may extend to other tissues as well. In view of the results of Neisser and Wechsberg, it was expected that this "bioscopic" method might be applied here to advantage.

Neisser and Wechsberg placed their mixture of cells, toxin, and methylene blue in small test-tubes, and covered the fluid with

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† *Münch. med. Wchnschr.*, 1900, 47, p. 1261; *Ztschr. für Hyg.*, 1901, 36, p. 330.

‡ *Trans. Chicago Path. Soc.*, 1903, 5, 207.

liquid paraffin to exclude the air, this being necessary for the accomplishment of reduction. I have found more convenient a tube, suggested by Professor Hektoen, of 4-5 mm. in diameter, drawn to a fine point at either end, which can be sealed in the flame after the tube is filled.

For the first experiments an emulsion of a guinea-pig brain in 30 c.c. of 0.85 per cent. NaCl was used, the tissue being ground fine with quartz sand, from which it was decanted; later a 5 per cent. emulsion was used uniformly. Aseptic precautions were used throughout and cultures made from the pipettes after the termination of the experiment. That contamination was infrequent may be due in part to bactericidal properties of the emulsion or of the methylene blue, or of both. Experiments which showed contamination were discarded. Reduction was allowed to take place at room temperature.

There is a quantitative relationship in the reduction of methylene blue by nervous tissue. In one case were used 0.5 c.c. of emulsion and varying quantities of methylene blue (0.1 drop to 16 drops of a methylene-blue solution which contained 5 c.c. of a saturated alcoholic solution of the dye and 95 c.c. sterile NaCl solution). One-tenth to one-half of a drop was completely reduced; in tubes with 0.5-5 drops partial reduction occurred, the color in succeeding tubes increasing gradually. In the remaining tubes the overlying fluid was densely blue, while the sediment and a very small zone above were decolorized after twenty-four hours. The fact that such an emulsion will reduce methylene blue at all probably indicates that intact cells are not essential. An emulsion kept in the ice-chest for a week will reduce, although less vigorously than when fresh. It occurred to Neisser and Wechsberg that this might be true, for they say: "It is entirely conceivable that certain cells may contain substances which in themselves have a reducing power, without regard to whether the cells as such are living or not."

The reduction is in no case permanent. Tubes after being completely reduced for three days regain the original color when shaken with air.

A drop of formalin added to 0.5 c.c. of the emulsion inhibited

reduction, so that at the end of three days there was only a trace, the control reducing completely in two hours.

Exposing the emulsion of nervous tissue to a temperature of 70° C. for thirty minutes destroys its reducing power (Table I). Tubes of the emulsion were treated as indicated in the table; then 1 drop of the methylene-blue solution was added.

TABLE I.
EFFECT OF HEAT ON THE REDUCING POWER.

50° C. for 30 min.	-	-	-	-	No retardation; complete in 2 hrs.
56° C. for 30 min.	-	-	-	-	Retarded; not complete in 10 hrs.
60° C. for 30 min.	-	-	-	-	Retarded; not complete in 10 hrs.
65° C. for 30 min.	-	-	-	-	Retarded; partial reduction at 18 hrs.: complete at 48 hrs.
70-95° C. for 30 min.	-	-	-	-	No reduction in 24 hrs.

Other experiments show that the emulsion also loses its reducing power when heated to 98° C. for five to ten minutes.

Subjecting the nervous tissue to the action of fresh (or activated) neurotoxic serum did not destroy, or even decrease, the reducing power of the emulsion. In Table II the mixtures were sealed at once without allowing time for a preliminary digestive(?) action of the serum upon the nervous tissue.

TABLE II.
THE INFLUENCE OF NEUROTOXIC SERUM ON THE REDUCING POWER OF NERVOUS TISSUE. SERUM OBTAINED FROM GOOSE AFTER IMMUNIZATION WITH NERVOUS TISSUE OF GUINEA PIG.

Emulsion	Serum	Meth. Blue Solution	
0.5 c.c.	0.5 c.c.	5 drops	Complete reduction in 18 hrs.
0.5 c.c.	0.4 c.c.	"	" " "
0.5 c.c.	0.3 c.c.	"	" " "
0.5 c.c.	0.2 c.c.	"	" " "
0.5 c.c.	0.1 c.c.	"	" " "
0.5 c.c.	0	"	" " "
0	0.5 c.c.	"	No reduction

Similar results were obtained when fresh rabbit serum was used in order to add complement, previous experiments having indicated that rabbit serum contains complement for the neurotoxic amboceptor of the goose serum.

Other experiments showed that the addition of neurotoxic

serum to the nervous-tissue emulsion intensified the reducing power of the latter, rather than inhibiting or destroying it. A tube containing 0.5 c.c. of serum reduced much more quickly than one containing 0.05 c.c. The serum alone is non-reducing.

Similar results were obtained by treating the nervous tissue with an immune rabbit serum. Digestion of the tissue for six hours with such a serum resulted in an increase in the reducing power, provided the serum was not removed by washing. If, however, the serum had been removed in this manner, the reducing power was decreased. For example:

a) Five c.c. of an emulsion two days old was centrifugated and the overlying fluid drawn off. One and a half c.c. of an active neurotoxin was then added to the sediment and the mixture kept at 37° C. for two hours, after which it was diluted to the original volume with salt solution.

Test 1.—One c.c. of *a)* + 1 drop methylene-blue solution: complete reduction in twelve to twenty-four hours.

Test 2.—*a)* was again washed, resulting in the removal of a large part of the serum: no reduction in three days.

b) Same as *a)*, except that normal rabbit serum was substituted for the neurotoxin.

Test 1.—Complete reduction.

Test 2.—No reduction in three days.

c) Same as *a)* and *b)*, except 0.85 per cent. sodium chloride solution was substituted for serum.

Test 1.—Partial reduction.

Test 2.—No reduction in three days.

d) Controls:

1. One c.c. of untreated emulsion reduced.

2. Two c.c. of pure rabbit serum gave no reduction.

It seems, then, that normal serum as well as the immune serum intensifies the reducing power. It is also evident that repeated washing of the emulsion—*c)*, 1 and 2—eliminates its reducing power, the presence of which in the original emulsion is indicated by Control 1. The conditions suggest that in the reduction of methylene blue by the nervous tissue and serum, as well as by the fresh emulsion without serum, there may be a co-operation of two substances, neither of which alone is reducing—*c)*, 2 and Control 2.

Inasmuch as repeated washing of an emulsion inactivated it,

it seemed probable that separation of the two suspected substances might be accomplished by extraction. Attempts at separation by centrifugating and washing a perfectly fresh emulsion were not successful; but after it had been allowed to extract for one to several days, one of the substances passed into the liquid portion in a state of solution or suspension, the other remaining incorporated with the solid tissue.

In Table III the emulsion had been kept in the ice-chest for eight days.

TABLE III.

SEPARATION OF THE REDUCING SUBSTANCES IN NERVOUS TISSUE BY EXTRACTION AND WASHING.

	24 hrs.	48 hrs.	72 hrs.
1. Unwashed emulsion.....	Partial reduc.	Reduced
2. Sediment washed three times.....	0	0	0
3. Original fluid of 1, after centrifugating.....	0	0	0
4. Washed sediment + 3 drops of guinea-pig serum	Reduced
5. Pure guinea-pig serum*	0	0	0

It appears, then, that the serum of guinea pig is able to replace the substance which was extracted from the tissue. Normal rabbit serum answers equally well for reactivation.

The extract† when added to washed sediment caused reduction, although either alone did not reduce. Reduction is not so vigorous, however, as when the original emulsion is used, probably because a portion of one or both substances is wasted in the intermediate washings. Filtering the extract through a Berkefeld filter removes the reactivating substance from the fluid. This would seem to indicate either that the substance is in large molecular form, or that it is associated with tissue particles in suspension which are held back by the filter.

The effect of successive washings (which means the progressive removal of the soluble substance) is seen in Table IV. Extraction was allowed to proceed for twenty-four hours in the refrigerator.

* In the course of several days pure serum causes partial reduction of the dye.

† By the "extract" is meant the fluid of a one to several days old emulsion after the sediment has been removed by centrifugation.

TABLE IV.

THE EFFECT OF REPEATED WASHINGS ON THE REDUCING POWER OF THE EMULSION.

	12 hours	24 hours	72 hours
Unwashed emulsion.....	Complete	Complete	Complete
Washed once—no serum.....	0	Trace reduction	Trace reduction
Washed once—serum, 2 drops....	Complete	Complete	Complete
Washed twice—no serum.....	0	Slight trace	Trace reduction
Washed twice—serum, 2 drops....	Partial reduction	Complete	Complete
Washed 3 times—no serum.....	0	0	0
Washed 3 times—serum, 2 drops....	Partial reduction	Almost complete	Complete
Two drops of serum alone.....	0	0	0

It is seen that as washing progresses there is a slight tardiness in the reactivation of the sediment by serum.

A fresh emulsion may be inactivated by heating to 98° for five to ten minutes, or to 70° for thirty minutes. The soluble substance is hereby destroyed, the one resident in the tissue sediment still being capable of reactivation by serum. The rapidity and completeness of reduction are proportionate to the quantity of serum used for reactivation. In Table V the emulsion was three and one-half days old, and for the experiment was heated to 98° for ten minutes in order to inactivate its reducing power. Guinea-pig serum four days old, was used to reactivate. The unheated emulsion reduced completely in twenty-four hours. Three drops of the serum alone did not reduce.

TABLE V.

THE ABILITY OF NORMAL SERUM OF GUINEA PIG TO REACTIVATE THE REDUCING POWER, AFTER THE LATTER HAD BEEN DESTROYED BY HEAT.

Emulsion	Serum	6 hours	24 hours	48 hours	72 hours
1.0 c.c.....	0	0	0	0	0
1.0 c.c.....	3 drops	Almost complete	Complete	Complete	Complete
1.0 c.c.....	2 drops	Partial			
1.0 c.c.....	1 drop	Trace	Almost complete	"	"
1.0 c.c.....	1/2 drop	0	Marked reduct'n		
1.0 c.c.....	1/4 drop	0	0	Partial	Marked
1.0 c.c.....	1/10 drop	0	0	Trace (?)	Trace

After five to eight days both substances may exist to some extent in the extract, since it is possible to render it reducing by the addition of serum.

In order to determine whether or not the reactivating power of serum depends on complement, guinea-pig serum was heated to different temperatures, and its reactivating power then tested (Table VI). In this experiment a 5 per cent. emulsion of nervous tissue was extracted for two days, then inactivated by three washings. The serum was from freshly drawn blood.

TABLE VI.

THE EFFECT OF HEAT UPON THE REACTIVATING POWER OF NORMAL SERUM OF GUINEA PIG.

Washed sediment (1.0 c.c. in each tube) to which was added 1, 2, and 3 drops of serum the latter having been heated to the different temperatures for thirty minutes.

The washed sediment in each case was suspended in the original volume of 0.85 per cent. NaCl.

Temp.	Observation Time	Serum, 1 Drop	Serum, 2 Drops	Serum, 3 Drops
40°	12 hours	0	0	0 (?)
	20 "	Almost complete	Complete	Complete
	33 "	Complete		
45°	12 "	0	0	Slight trace
	20 "	Almost complete	Complete	Complete
	33 "	Complete		
50°	12 "	0	Trace	Trace
	20 "	Complete	Complete	Complete
	33 "			
55°	12 "	0	Partial	Almost complete
	20 "	Complete	Complete	Complete
	33 "			
60°	12 "	0	Marked	Almost complete
	20 "	Partial (!)	Complete	Complete
	33 "	Partial (!)		
65°	12 "	Trace	Almost complete	"
	20 "	Complete	Complete	"
	33 "			"
70°	12 "	Almost complete	Almost complete	"
	20 "	Complete	Complete	"
	33 "			"

Controls.—(1) Unaltered emulsion: complete reduction in twelve hours.

(2) Extract: no reduction in twenty-four hours.

(3) Washed sediment; no reduction in thirty-six hours.

(4) Washed sediment + fresh, unheated guinea-pig serum:

	1 Drop	2 Drops	3 Drops
Twelve hours.....	0	Trace	Marked
Twenty hours.....	Partial	Complete	Complete
Thirty-three hours.....	Complete		

(5) 3 drops of serum heated to 40-70° C. for thirty minutes showed no reduction at thirty-three hours.

In this experiment it is seen that the heating of the serum, rather than destroying the reactivating power, has increased it, in which respect there is no resemblance to ordinary complement. It is also different from the "soluble" substance of the extract as a reactivator, since the latter is destroyed by a temperature of 70° for thirty minutes. A difference from ordinary complement is also shown by the fact that an old serum will serve to reactivate. Temperatures higher than 70° were not tried.

Influence of acids and alkalies.—Hydrochloric acid, except when an exceedingly minute amount is added, destroys the reducing powers of the fresh emulsion. It will not reactivate an inactive sediment. Quantities of 0.1–0.2 c.c. of normal potassium hydrate solution will reactivate the boiled emulsion or the sediment which has been made inactive by washing. Smaller quantities of the alkali have a noticeable, but weaker effect. Potassium hydrate alone does not reduce methylene blue, although it affects the dye chemically, as shown by the appearance of a lavender color.

Influence of tetanus toxin.—Tetanus toxin in any quantity does not affect the reducing power of nervous tissue for methylene blue.

SUMMARY AND CONCLUSIONS.

The "biscopic" method proved unsuitable as a means of determining the toxic action of neurotoxic serum, owing possibly to the fact that this serum, in common with normal serums, contains properties adjuvant to the reduction of methylene blue.

In the reduction of methylene blue by nervous tissue the living cell is not essential.

Reduction by the tissue emulsion is accomplished by the combined action of two substances, one of which, a thermolabile substance, may be extracted from the tissue by a 0.85 per cent. NaCl solution; the other, a thermostabile substance, is closely associated with the solid tissue.

Serum, whether it is old, fresh, or heated to 70° for thirty minutes, may be substituted for the thermolabile substance, as also may potassium hydrate.

Chemists have determined that the reduction of methylene blue

may be accomplished only by the action of nascent hydrogen on the dye. It becomes leuco-methylene blue when its affinities for hydrogen have been satisfied.*

In the original emulsion this hydrogen may have its source in fermentation processes (glycolytic or proteolytic), since certain ferments and the substances which they specifically affect are known to exist normally in tissues and body fluids.

When serum is used as a reactivator, it is questionable if the reduction can be referred to the action of ordinary ferments in view of the heat resistance of the substance contained in the serum (70° for thirty minutes). The serum may contain, in addition to ferments, some obscure catalyzing agent which acts chemically upon substances contained in the tissue.

It would seem that the action of potassium hydrate must be referred to its catalyzing properties.

It has not been possible to determine the amboceptor and complement nature of the reaction under discussion, since the binding phenomenon could not be demonstrated; however, a degree of analogy is not lacking.

A few experiments performed with 5 per cent. emulsions of liver and kidney tissues indicate that similar principles prevail in the reduction of methylene blue by these tissues.

Although a most interesting chemical problem is concerned in the phenomenon presented, one is confronted with so many unknown substances and reactions that the writer has not thought it profitable at present to follow this work further. It is not unlikely that methylene blue may be made to play the part of an indicator of reduction reactions where sterile substances can be dealt with.

*Boiling a solution of glucose and sodium carbonate which contains methylene blue, or the hydrogen evolved by the action of a mineral acid on tin, in a methylene-blue solution, accomplish the reduction of the dye. Potassium ferrieyanide reduces, the ferrocyanide oxidizes, the dye.

THE ANTAGONISM EXHIBITED BY CERTAIN SAPROPHYTIC BACTERIA AGAINST THE BACILLUS TYPHOSUS GAFFKY.*

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INTRODUCTION.

THE effect which various agencies have on the life of *Bacillus typhosus* outside of the human body is a subject of great hygienic importance. Any information which will throw light on this subject is sure to receive due consideration.

It is held by some observers that this organism never finds conditions favorable for its development outside of the body, and that its existence in nature is measured by a few days, or at most a few weeks. Others, on the other hand, believe that this germ may lead a saprophytic existence, and that its life in nature may be prolonged indefinitely.

Whichever view may be ultimately shown to be correct, it is certainly true that the extracorporeal sojourn of the typhoid germ is influenced by certain factors. *e. g.*, the nature of the food substances, the amount of moisture, etc. The effect of these various factors has been repeatedly studied, and it may be presumed that, in a general way, their influence is fairly well understood.

There are, however, a number of factors whose influence is more or less uncertain. Of these perhaps none is more important or worthy of more careful consideration than the effect which various other bacteria have on the typhoid germ in their association with it.

Bacteria in nature occur almost invariably in mixed cultures. Their association may be without effect on the various species, or it may affect them in various ways. They may offer mutual or one-sided aid, and thus live in a symbiotic relation. They may, on the other hand, offer mutual or one-sided injury, *i. e.*, they may exert an antagonism on one another.

The present paper is a record of experiments performed for the purpose of determining the effect of the association of other bacteria on the typhoid germ. The bacteria studied are those which the typhoid germ would be likely to meet with in nature. All of them are well-known saprophytes.

Where an antagonism has been shown to be exerted an attempt has been made to determine the laws which govern this antagonism.

HISTORICAL REVIEW.

The fact that certain bacteria are antagonistic in their action toward *B. typhosus* has been known for many years.

v. Freudenreich¹ in 1888 determined that when certain bacteria were grown in flasks of broth for some time, and then filtered through a porcelain filter, the typhoid germ failed to grow in the filtrate in some cases, and in other filtrates it grew only feebly. The organisms which allowed the typhoid germ to grow feebly in its by-products were *Staphylococcus pyogenes aureus*, *B. typhosus*, *Bacillus* of chicken cholera, *Spirillum* of asiatic cholera, *Spirillum* of Miller, and *Spirillum* of Denecke. Those which did not permit the growth of the typhoid bacillus were: *Staphylococcus pyogenes albus*, *Staphylococcus pyogenes fetidus*, *B. pyocyaneus*, and *B. phosphorescens*; while in the case of *Bacillus* of symptomatic anthrax its growth was only delayed, and in the filtered cultures of *Spirillum* of Finkler and Prior it grew well.

Garré² in 1888 showed that *Pseudomonas fluorescens putida* produces in its growth on artificial media substances which are antagonistic to the typhoid germ. He grew this organism on gelatin, and then scraped it off and seeded the typhoid germ, and found that it would not grow, although *Ps. fluorescens putida* would grow on media from which a growth of *B. typhosus* had been scraped. He also demonstrated the existence of an antagonism by means of alternate streaks of the two organisms arranged radially on a gelatin plate. Where the streaks were near together the typhoid germ did not grow, but near the circumference, where the distance between the streaks was greater, both organisms developed normally (Fig. 1).



FIG. 1.—Drawing illustrating Garré's method of studying antagonism—alternate streaks. The large streaks of *Ps. fluorescens* were made and allowed to grow twenty-four hours, and then streaks of *B. typhosus* were made which grew only at the circumference of the plate.

Olitzky³ in 1891 worked on the antagonism which is exerted by *B. fluorescens liquefaciens* (*Pseudomonas fluorescens*), employing methods slightly modified from those of Garré.² This observer demonstrated a marked antagonism and laid stress on the hygienic importance of this fact.

Laws and Andrewes⁴ in 1894, working on the duration of the life of *B. typhosus* in sewage, appeared to show that the presence in the sewage of *B. fluorescens liquefaciens*, and especially *B. fluorescens stercoralis*, shortened the life of the typhoid germ.

Sidney Martin⁵ in 1898-1900, studying the growth of the typhoid bacillus in soil, determined that, while some of the soils furnished conditions favorable for the prolonged existence of the typhoid germ—in one case as long as 456 days—other soils presented conditions which were inimical to the growth

of the typhoid bacillus. This antagonism of certain soils he was able to trace to the effect of definite bacteria which he isolated and grew in pure culture. The identification of these bacteria was not established, but they were described as "Chichester 1, 2, 3, 4, and 5." The antagonism was tested by growing the organisms separately with the typhoid germ in 200 c.c. of sterile water to which 10 c.c. of sterile broth had been added. With No. 1 the typhoid bacillus died in less than twelve days at 8-12°, and six days at 37°. No. 5 killed out the typhoid germ in less than seven days at 37°. When these organisms were grown with the typhoid germ in sterile soil, No. 1 had gained the upper hand in twelve days at 8-12°, and in six days at 37°; No. 3, in fifteen days, and No. 5 in three days at 37°.

Rémy⁶ in 1901 writes on the antagonism exhibited by *B. coli* for *B. typhosus*. He was, however, unable to show any antagonism, but maintained that the specific characters of both germs were changed. This change was in respect to their agglutination reaction as well as their cultural characters.

Horrocks⁷ in 1901, working with *Pseudomonas fluorescens*, found that *B. typhosus* would not grow on gelatin which had already yielded a growth of *Ps. fluorescens*, but that gelatin which had served as a medium for *B. typhosus* would still permit the development of *Ps. fluorescens*. In sterile sewage he was unable to obtain *B. typhosus* after it had grown seven days with *Ps. fluorescens*. Working also on the effect on *B. typhosus* of its association with *B. coli*, with what he considered improved methods, namely, alkaline, glucose-litmus agar surface plates, he found that when these two organisms were grown together in sterile tap water *B. typhosus* could be isolated after twenty-one days, but in peptone water it could not be discovered after seven days.

METHODS AND MATERIALS USED.

METHODS USED.

A considerable number of methods have already been described by others for demonstrating the action which one organism may exert on another. It is proposed in this section briefly to describe these methods, and also, somewhat more in detail, the original methods which are here employed.

1. *Simultaneous culture on solid media*.—Garré was the first to use this method. As used by him, it consists of making streaks on the surface of agar or gelatin plates of the two organisms to be studied. The streaks are alternate, and may be either parallel, or radiating from a common center, or they may intersect at right angles. In all cases the streaks of the two organisms alternate. (Fig. 1 shows this method.)

In cases where the antagonistic substance does not diffuse for any considerable distance into the medium it is not satisfactory.

In this work the method has been modified in some instances so as to overcome this, as follows: The medium was seeded with *B. typhosus*, and when it had hardened, the opposing bacterium

was planted by making one or more streaks across the surface. (See Fig. 2.)

2. *Successive cultures on solid media (Garré).*—Here one of the organisms is allowed to originate a good growth on a solid medium, as gelatin or agar. Then this growth is scraped off, and the second organism is sown on the medium. This is a useful method, and one that has not infrequently been used.



FIG. 2.—Drawing showing a modification of Garré's method. Agar plate heavily seeded with *B. typhosus*, and then streaked with *B. vulgaris*. Colonies are very small under and near the streak.

3. *Culture in filtered by-products.*—v. Freudénreich¹ grew one of the organisms to be studied in broth, and after some time filtered the medium through a porcelain Pasteur filter, and then seeded this sterile filtrate with the other organism, and by means of plate cultures, or otherwise, determined the behavior of this organism in the by-products of the other.

4. *Cultures on opposite sides of porcelain filter.*—Frankland and Ward⁸ used a porcelain filter of the Pasteur-Chamberland system, partially filled with broth and arranged in a beaker of broth. (See Fig. 3.) The two germs to be experimented with are sown on opposite sides of the filter, where their behavior can be readily ascertained. Theoretically this method is ideal; practically, however, it is open to the objection that usually, if not invariably, motile bacteria will grow through the porcelain after a time. Indeed, a similar arrangement has been suggested for the separation of *B. typhosus* from the less motile *B. coli*. See in this connection Cambier,⁹ and also von

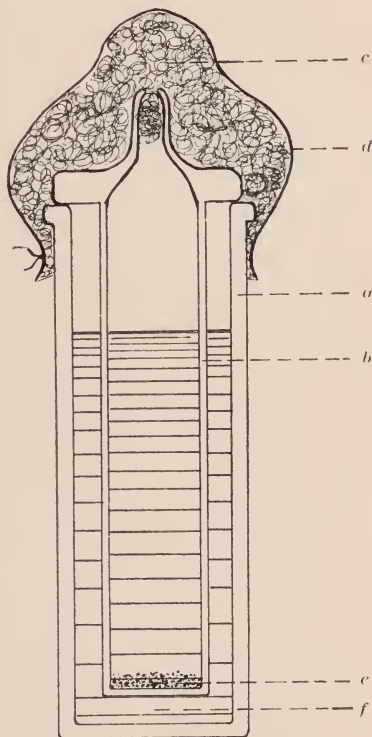


FIG. 3.—Frankland and Ward's method of studying antagonism. *a*, a glass jar; *b*, porcelain filter, Pasteur-Chamberland system; *c*, cotton wool; *d*, paper cap; *e* and *f*, broth in which are growing the two antibiotics.

Esmarch¹⁰ who gives figures showing the bacteria in the pores of the filter on their way through.

5. *Collodion sac method*.—Collodion sacs are formed as described in a former paper (Frost¹¹). The tops are trimmed, and then they are drawn over test-tubes, from which the bottoms have been cut, fastened on with a thread or rubber band, and sealed with a little fresh collodion. The tube thus prepared is then partially filled with broth and placed in a small flask of broth. The test-tube with the collodion sac on the end is held in place in the flask by a packing of cotton. They are sterilized in the steamer or autoclave.

In this work the medium inside the sac has been seeded with *B. typhosus*, and the opposing germs have been placed in the flask. (See Fig. 4.) An arrangement similar to this was described by Ruffer and Crendiropoulo¹² in 1900. Results obtained by this method do not seem to have been published, and the arrangement described here had been in use for some months before their description was seen.

6. *Double-plate method (original)*.—In this method a Petri dish is divided into two halves by means of a small glass tube or rod. After sterilization it is used by seeding an agar tube rather heavily with one of the organisms to be tested, and then pouring it into one-half of the plate. When this has hardened, a tube of sterile, uninoculated agar is poured into the other half. When this in turn is hardened, the other organism (in this work *B. typhosus*) is streaked across the surface of both halves, the seeded and the sterile. It is necessary, however, in order that the streak on both sides be equally heavily seeded, to streak each side separately. This is best done by having a loop bent at nearly right angles, and then with the charged loop to begin at the circumference and streak to the glass rod, sterilize and recharge the needle, and then continue the streak on the other side of the plate. In this way two parallel streaks are made across the plate. The streaks may be made as soon as the other organism has been planted, or as is usually desirable, the organism in the seeded half of the plate may be given an opportunity to develop before the other is streaked, thus making the antagonism more striking. (See Plate XVIII, Fig. 1.)

7. *Agar-block method (original)*.—An agar jelly is made by dissolving 2 per cent. of agar-agar threads in distilled water, filtering through filter paper, and sterilizing. This agar is then



FIG. 4.—Collodion sac as arranged for the study of antagonism. The *Bacillus typhosus* is in the broth in the sac, while the opposing organisms are outside in the broth in the flask.

poured into a deep Petri dish or a crystallizing dish; the purpose being to obtain a cake of agar about an inch and a quarter in thickness. With a knife which has been sterilized in the flame the solidified agar is cut into blocks about two-fifths of an inch square and the height of the block. These blocks are seeded by stabbing them with a platinum needle charged with one of the organisms to be tested, in the same way that an ordinary stab culture is made. Care should be taken not to make the stab quite to the bottom of the block. The top of the block is sealed by touching it with a red-hot iron, *e. g.*, the head of a nail. In this way the bacteria which are left on the surface at the time of inoculation are killed, and when the agar cools the seal is complete. With a little practice blocks can be readily made so that their outer surface is perfectly sterile. These blocks are then placed by means of a pair of sterile forceps, in a broth test-tube culture of the other organism. When such blocks are placed in a culture medium, sufficient nutritive material diffuses through the layer of agar so that growth occurs along the line of puncture. In the case of sterile broth a heavy growth appears in the course of a few days. When these blocks are put in cultures, if any antagonistic factors are operative, growth may be only slight or not at all apparent. (See Fig. 5 and Plate XVIII, Fig. 2.)

MEDIA USED.

All the ordinary media used were made according to the procedures recommended by the Bacteriological Committee¹³ and had a reaction of +1 (Fuller's scale). In a number of the experiments some of the differential media were used, *e. g.*, carbolized agar as used by Chick¹⁴ and Hiss's¹⁵ new media, but soon given up, as in the frequently crowded plates, and with cultures of *B. typhosus* which had been kept on artificial media for some time, there was no constant characteristic. All of the media were made from extract of beef and were sterilized in the autoclave at a temperature of 120°.

CULTURES OF BACILLUS TYPHOSUS USED.

Three cultures have been used. One, known in the laboratory as the M. H. culture, was received in 1898 from the United States Marine Hospital Service Laboratory, and since then has been used

in the laboratory as the standard culture, and was such when these researches were begun. Later, however, the organism began to show a tendency to clump in ordinary broth cultures, and it was then discarded for the second culture. This is designated as the P. D. 1. This was obtained in the fall of 1902 from Parke, Davis & Co., of Detroit, Mich. This gave a typical reaction with typhoid blood. A third culture has been used to some extent, P. D. 2. This was obtained from the same source about six months later.

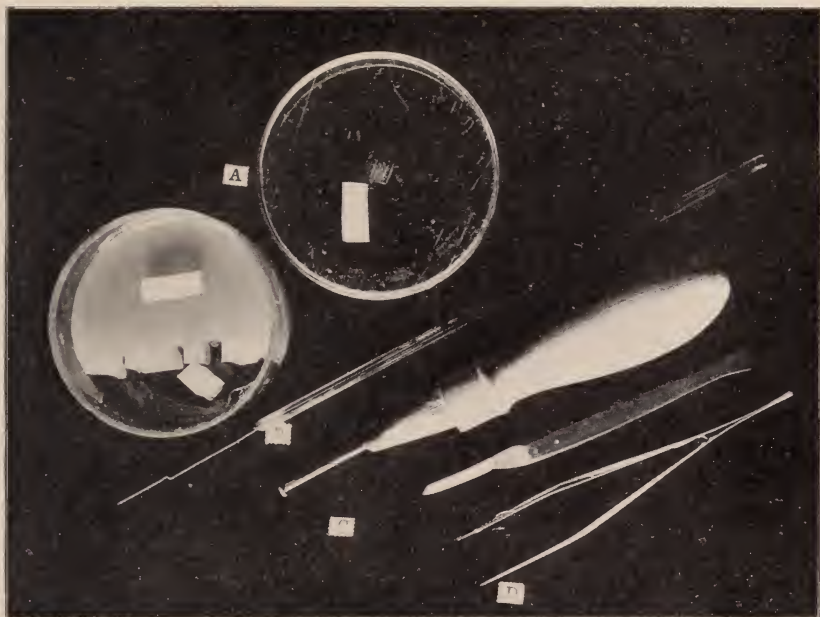


FIG. 5.—Method of making agar blocks. A, deep Petri dish and cover showing how blocks are cut out, also two on side; B, needle arranged to prevent making too deep stab; C, nail in handle for sealing end of block; D, forceps for handling blocks.

ANTAGONISM EXHIBITED BY VARIOUS MIXED CULTURES OF BACTERIA AS TESTED BY MEANS OF COLLODION SACS.

In studying the effect of the association of other bacteria on *B. typhosus*, one of the chief difficulties has always been the inability of recovering *B. typhosus* from such mixtures with ease and certainty. The use of selective media, especially those of Elsner and Hiss,¹⁵ has given promise of success, but in my own

hands, at least, has proved to be of uncertain value, since the characteristic features of the typhoid on these media appear to become weakened in old laboratory cultures and are frequently closely simulated by other germs.

It would seem that ideal conditions would be reached in the study of the association of bacteria if one of the germs could be inclosed in a receptacle protecting it from intimate association with the other germs, but permitting the ready interchange of the nutritive solutions and by-products. Under these conditions the by-products produced by the germs growing on one side of the walls of the receptacle would presumably reach the germs on the other side, while they would at the same time be kept in pure culture and could be examined at will. With these conditions the effect of association ought to be as accurately determined as if the opposing bacteria were in actual contact, for at the present time we can conceive of the action of any germ exerted against another only as being accomplished by means of chemical poisons; and if the walls of the receptacle permit of a ready exchange of these, the conditions would be thoroughly satisfied.

The use of the collodion sac would seem to offer the desired means. It has been used so successfully in other work, especially with animals, that it seemed likely to be valuable in this connection.

METHODS.

The collodion sacs were made and arranged as previously described (see p. 604). The medium used outside the sac was ordinary broth, that within the sac was either broth or sterile tap water. The water used in this case was tap water drawn from Lake Mendota, Wis. This water has a rather high bacterial content. When used, the water was sterilized in the autoclave.

The medium outside of the sac was seeded with material containing, presumably, various kinds of bacteria, and *B. typhosus* was inoculated into the medium within the sac. In some instances the seeding of *B. typhosus* and the opposing germs was simultaneous; in other cases the seeding of *B. typhosus* was delayed until the bacteria outside of the sac had grown for some time. The start allowed the opposing germs varied from a few hours to

several days. In all cases the medium within the sac was seeded heavily with *B. typhosus*, for the reason that it seems certain, from a considerable series of observations in another connection, that surroundings which inhibit the growth, or actually kill, *B. typhosus* when it is present in small numbers are frequently inoperative when the seeding is heavy. In all cases in the following tables the figures refer to the number of germs present in a single loopful, the loop being one of the standard size recommended in the Procedures of the Bacteriological Committee.¹³ The temperature at which the experiments were performed was that of the laboratory, 18–20° C., or in an incubator at 28° C. In the various experiments the temperature used is indicated.

The periods elapsing between the various quantitative determinations are not constant. Usually a number of determinations were made: in some cases they were few, but at the same time were considered sufficient for the purpose in hand.

SERIES I. SOIL BACTERIA.

This series was of a preliminary nature. It served to test the methods and suggested the succeeding work. The medium was broth and that in the flask was inoculated with a sample of soil obtained from the university campus. Flask No. 2 was not inoculated outside of the sac and served as a control. The culture of *B. typhosus* used was the United States Marine Hospital culture (M. H.). The temperature was that of the laboratory in summer, *i. e.*, about 22° C.

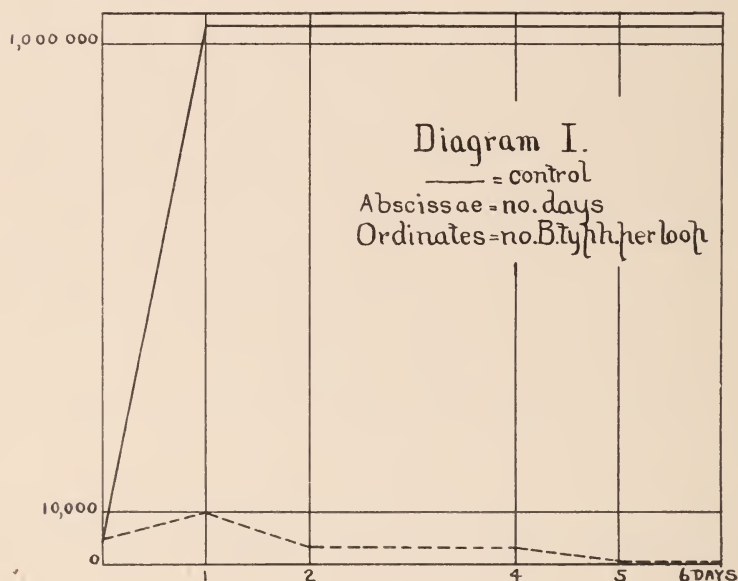
The data obtained are exhibited in the following table (Table I). It is also arranged graphically in Diagram I. The vertical distances, or ordinates, represent the number of typhoid germs per loop of medium. The horizontal distances, or abscissæ, represent the days. In regard to the meaning of the infinity sign used in the table see footnote to Table I.

TABLE I.
EFFECT OF SOIL ORGANISMS ON *B. TYPHOSUS*.

NO. OF EX- PERI- MENT	SOURCE OF ORGAN- ISMS		PERIOD OF PRELIM. CULTIVA- TION		TEM- PERA- TURE	NO. OF COLONIES OF <i>B. TYPHOSUS</i> DEVEL- OPING ON AGAR PLATES FROM ONE LOOP OF THE MEDIUM							RE- SULT
	Opp.	B. ty.	Opp.	B. ty.		0	1 D.	2 D.	4 D.	5 D.	6 D.		
1.....	Soil	M. H.	0	0	20-22	5,290	9,600	3,300	2,900	200	100	..	- 98 %
2 (con- trol)	M. H.	..	0	20-22	4,900	∞*	∞	∞	∞	∞	..	+ ..

* The infinity sign (∞) means here that they could not be counted with a simple microscope. Later the compound microscope was used, and actual figures were given where the numbers were as great, possibly, as above.

The antagonism of these soil bacteria exerted against the typhoid germ is very strongly marked here; for while the experiments were not continued until the typhoid germ had entirely disappeared, it is to be noted that in the six days 98 per cent. of *B. typhosus* had been killed, and there is no doubt that in the course of a few days more they would have entirely disappeared. On the other hand, *B. typhosus* would live, as is well known, not only for many days, but for months, under conditions less favorable than those of the control culture.



SERIES II. SOIL BACTERIA.

This series was undertaken to confirm the results of the preceding experiment, and also to extend the observations on the extent of the distribution of the antagonistic organisms. The media outside of the sacs were inoculated with small amounts of soil from various sources which were selected with the idea of securing a variety of different micro-organisms.

Soil No. 1 was taken near the well at South Hall on the University campus. This well is frequently used, and the soil in the immediate neighborhood is kept constantly moist.

Soil No. 2 was obtained in the dust from the macadam road leading to Main Hall. This road is comparatively little used.

Soil No. 3 was taken from the woods north of Main Hall. This is a typical wood soil. It is a virgin soil.

Soil No. 4 was sand brought directly from a pit, and was being used to make a cement walk.

It will thus be seen that, while the soils all differ very materially, they are all to be classed as uncontaminated soils, except, possibly, sample No. 2, and probably not fitted to furnish conditions favorable for the development of *B. typhosus*. With the exception of sample No. 1, they are all dry soils.

The broth was inoculated, and the cultures were grown for six days, when it was intended to inoculate the sacs with *B. typhosus*. It was found, however, at this time that all of the sacs were imperfect and had become contaminated. They were then removed and replaced with sacs which had been tested by growing *B. typhosus* in them for twenty-four hours. It thus occurred that the soil bacteria had had opportunity for abundant development before *B. typhosus* was brought in contact with them. But it should also be noted that the sacs were very thickly seeded, and thus *B. typhosus* would be less easily affected. The temperature of this experiment was 28° and the typhoid culture the M. H.

The results are shown in Table II:

TABLE II.
EFFECT OF SOIL ORGANISMS ON *B. TYPHOSUS*.

NO. OF EX- PERI- MENT	SOURCE OF ORGAN- ISMS		PERIOD OF PRELIM. CULTIVA- TION		TEM- PERA- TURE	NO. OF COLONIES OF <i>B. TYPHOSUS</i> DEVEL- OPING ON AGAR PLATES FROM ONE LOOP OF THE MEDIUM						RE- SULT
	Opp.	B. ty.	Opp.	B. ty.		0	1 D.	3 D.	5 D.			
1.....	Soil 1	M. H.	6 days	1 day	28	∞ *	∞	10	25	-100 %
2.....	" 2	"	6 "	1 "	28	1,100	∞	200	10	-100 %
3.....	" 3	"	6 "	1 "	28	∞	∞	∞	6	-100 %
4.....	" 4	"	6 "	1 "	28	∞	∞	10	25	-100 %
No.	control											

The antagonism exerted here is more marked even than in the preceding series, being practically 100 per cent. decrease in five days. This result is obtained in spite of the fact that the sacs were very heavily seeded to start with. It is to be noted here also that the infinity sign has a special meaning, as explained in footnote to Table I.

SERIES III. SOIL BACTERIA.

In this series the broth in the flasks was inoculated with the soil, and the bacteria were allowed to grow twelve days. In order to be sure that the new set of sacs were tight, they were inoculated, as in the previous series, with *B. typhosus*, and grown in sterile broth for forty-eight hours. Those sacs which remained tight were then transferred to the flasks prepared above: so that we have in this series typhoid cultures two days old, and hence

* See footnote to Table I.

thickly seeded, in collodion sacs submerged in flasks containing cultures of mixed soil bacteria grown for twelve days in broth. The samples of soil used are the same as in the previous series. The culture of *B. typhosus* used here is not the same as that used before, but is the strain described above as *B. typhosus*, P. D. 1.

The temperature of the experiment was 28°.

TABLE III.
EFFECT OF SOIL ORGANISMS ON *B. TYPHOSUS*.

NO. OF EX- PERI- MENT	SOURCE OF ORGANISMS		PERIOD OF PRELIM. CULTIVATION		TEMPERATURE	NO. OF COLONIES OF <i>B. TYPHOSUS</i> DEVELOPING ON AGAR PLATES FROM ONE LOOP OF THE MEDIUM							RE- SULT
	Opp.	B. ty.	Opp.	B. ty.		0	1 D.	2 Days	3 D.	4 D.	5 D.	6 D.	
1.....	Soil 1	P.D.1	12 d.	2 d.	28 C.	61,000	..	1,440,000	2,000	-96%
2.....	" 2	" 1	12 "	2 "	28 "	76,000	..	4,000	14	-99%
3.....	" 3	" 1	12 "	2 "	28 "	172,000	..	36,000	4,000	-98%
Con- trol	..	" 1	..	2 "	28 "	40,000	..	∞	∞	+

In this series the soil bacteria had been given abundant opportunity to develop their by-products before *B. typhosus* was introduced. It is to be borne in mind, however, that there was little or no gain experienced by the extra six days, since it is hardly likely that any considerable amount of growth would have taken place in this last week. It seems probable, then, that the antagonistic action would not necessarily be more marked than in the previous series, even if the other conditions had been identical.

The sacs did not contain any more bacteria per loop in this case than in the preceding case, although the cultures were a day older when brought in contact with their antagonists. It is further to be remembered that the infinity sign has a special and limited meaning.

Thus, while the antagonism exerted by the opposing bacteria is not as marked as in the preceding experiments, it is very apparent and in all cases to be represented numerically as about 95 per cent.

The change in the strain of *B. typhosus* used may also explain the apparently lessened effect of the antagonism.

SERIES IV. SOIL BACTERIA.

This series was undertaken for the purpose of extending the range of observations on the antagonistic action of soil bacteria to other samples of soils. The source of the material is given here.

Soil No. 5 is from the woods north of Main Hall on the campus, and is the same as No. 3 in Series II and III, except that it was collected late in the fall instead of in the summer.

Soil No. 6 is from a field on the university farm south of Observatory Hill. This soil has been under cultivation several years, and had been plowed in the fall before the sample was taken.

Soil No. 7 is clay excavated in the construction of Agricultural Hall.

Soil No. 8 is a sample of dirt from a macadamized drive running along the university grounds (Linden Drive).

Soil No. 9 is from the back yard of a dwelling-house. (This is "made soil" which for several years had been contaminated with house refuse, the burial of garbage, and horse manure.

Soil No. 10, sand being used for mortar, obtained directly from the pit. Presumably this is like No. 4 in Series III and IV.

The technique in this series was the same as in the preceding experiments.

B. typhosus was inoculated into the sacs, and allowed to grow twenty-four hours before being brought in contact with the opposing germs.

The opposing germs were inoculated at the time the sacs were introduced, and were not given any time for preliminary development.

The culture of the typhoid germ is the same as in the preceding series, viz., the P. D. I. culture.

The temperature of the experiment is 28°.

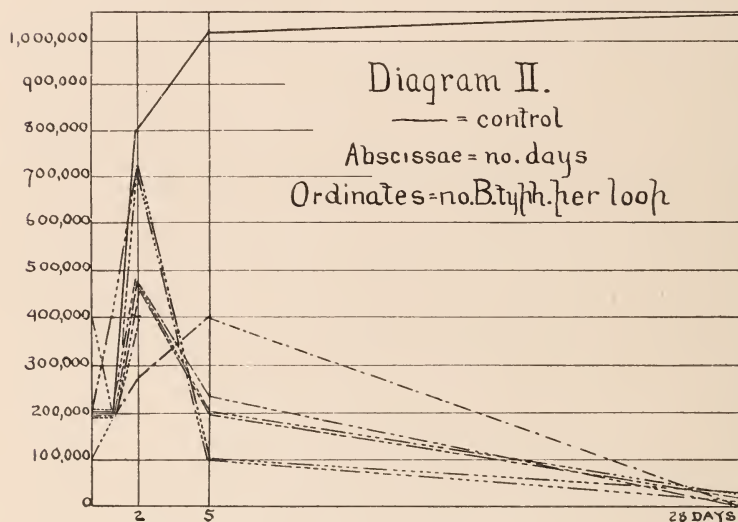
The results are shown in Table IV, and graphically in Diagram 2.

TABLE IV.
EFFECT OF SOIL ORGANISMS ON *B. TYPHOSUS*.

NO. OF EX- PERI- MENT	SOURCE OF ORGANISMS		PERIOD OF PRELIM. CULTIVA- TION		TEMPERATURE	NO. OF COLONIES OF <i>B. TYPHOSUS</i> DEVELOPING IN AGAR FROM ONE LOOP OF THE MEDIUM						RE- SULT
	Opp.	B. ty.	Opp.	B. ty.		0	1 Day	2 Days	5 Days	28 Days	
1.....	Soil 5	P.D.I	0	1 day	28° C.	200,000	200,000	28,000	400,000	0	- 100%
2.....	" 6	" 1	0	1 "	28	200,000	200,000	480,000	240,000	0	- 100%
3.....	" 7	" 1	0	1 "	28	200,000	200,000	480,000	200,000	200	- 99%
4.....	" 8	" 1	0	1 "	28	400,000	200,000	480,000	200,000	200	- 99%
5.....	" 9	" 1	0	1 "	28	200,000	400,000	720,000	100,000	-100	- 99%
6.....	" 10	" 1	0	1 "	28	100,000	200,000	720,000	100,000	200	- 99%
Control	" 1	1 "	28	200,000	200,000	800,000	∞	∞	+ 99%

In this series *B. typhosus* was given every opportunity to maintain its own or even to grow; still it showed a decline which finally amounted to 99 per cent. in all of the samples. The time required to bring this about was considerably greater than in the previous experiments. It is unfortunate that there were no counts

between the fifth and the thirtieth days, so that the exact rate of the decline could have been determined; for it will be noticed that on the fifth day no decrease was observable; indeed in some cases (1 and 2) there appeared to be an actual increase. After the elapse of thirty days the decrease was marked, and in striking contrast to the condition of affairs in the control, where there was no decrease to be detected after a month's time.



SERIES V. SOIL BACTERIA.

The purpose of this series was to retest the soils used in the previous experiment under conditions such that the opposing bacteria would have an opportunity to produce their antagonistic properties before *B. typhosus* was introduced.

The media in the flasks were inoculated with the various soils and allowed to grow for twelve days. At this time saes were introduced which had been inoculated with *B. typhosus* two days before. It thus occurred that *B. typhosus*, grown for two days in sterile broth, was brought in contact with the opposing bacteria which had grown for twelve days.

The soils were the same as those used in the previous experiment, viz., Nos. 5-10.

The temperature was 28°, as usual.

The culture of *B. typhosus* used was P. D. 1.

TABLE V.
EFFECT OF SOIL ORGANISMS ON *B. TYPHOSUS*.

No.	SOURCE OF ORGANISMS		PERIOD OF PRELIM. CULTIVATION		TEMPERATURE	No. OF COLONIES OF <i>B. TYPHOSUS</i> DEVELOPING ON AGAR FROM ONE LOOP OF THE MEDIUM						RE- SULT - Inc. + Dec.
	Opp.	B. ty.	Opp.	B. ty.		0	1 Day	2 Days	3 Days			
1.....	Soil 5	P.D.2	12 d.	2 d.	28 C.	480,000	40,000	0	-100%
2.....	" 6	" 2	12 "	2 "	28 "	40,000	4,000	3,600	- 90%
3.....	" 7	" 2	12 "	2 "	28 "	240,000	20,000	0	-100.
4.....	" 8	" 2	12 "	2 "	28 "	480,000	240,000	4,000	- 99%
5.....	" 9	" 2	12 "	2 "	28 "	48,000	32,000	∞ ¹	+
6.....	" 10	" 2	12 "	2 "	28 "	co	ntaminated		
Control	" 2	2 "	28 "	440,000	360,000	∞	+

In a general way the results obtained here are what were to be expected, viz., that when the soil bacteria are given a chance to develop their by-products, and then *B. typhosus* is brought in contact with them, the result is a marked and rapid decrease in *B. typhosus*.

In Nos. 1 and 3 (Table V) *B. typhosus* was entirely killed off in the amount tested. In Nos. 2 and 4 the decrease was marked, but not carried to the extreme as in the above cases.

No. 5 seems to be an exception, and behaves as the controls have behaved heretofore. This is probably accidental, since with the same soil in the previous experiment there is no marked contrast with the other soils, although it is to be noted that this soil is the only one which might be considered a contaminated soil.

SERIES VI. WATER BACTERIA.

In this series the broth is inoculated with water organisms instead of soil organisms.

The water used in one case is that of Lake Mendota, and in the other case a cistern from a private residence.

No opportunity was given the water bacteria to produce their poisons in the broth before the sowing of *B. typhosus*; indeed, the *B. typhosus* was inoculated twenty-four hours in advance of the other organisms.

The culture of *B. typhosus* used was that of P. D. 1.

The temperature was that of 23°.

The results are shown in Table VI:

¹ Probably a contamination.

TABLE VI.
EFFECT OF WATER ORGANISMS ON *B. TYPHOSUS*.

No.	SOURCE OF ORGANISMS		PERIOD OF PRELIM. CULTIVATION		TEMPERATURE	No. OF COLONIES OF <i>B. TYPHOSUS</i> DEVELOPING IN AGAR FROM ONE LOOP OF THE MEDIUM						RESULT + Inc. - Dec.
	Opp.	B. ty.	Opp.	B. ty.		0	1 Day	2 Days	5 Days	6 Days	30 Days	
1.....	(Water) Lake	P.D.1	0 d.	1 d.	28° C.	200,000	200,000	400,000	400,000	0	-100%
2.....	Cistern	" 1	0 "	1 "	28° "	200,000	200,000	800,000	400,000	0	-100%
3.....	Lake	" 1	12 "	2 "	28° "	300,000	640,000	64,000	- 78%
4.....	"	" 1	12 "	2 "	28° "	120,000	64,000	64,000	- 47%
5.....	"	M. H.	0	0	28° "	5,200	9,600	3,300	200	100	0	-100%
Control	P.D.1	28° "	200,000	200,000	∞	∞	∞	∞	+

It would appear here, as in the previous experiments on soil bacteria, that *B. typhosus* is at a distinct disadvantage when grown under conditions which permit the action on it of the by-products of certain other bacteria found in nature.

That this antagonism is not immediately demonstrable is undoubtedly due to the fact that *B. typhosus* was given an opportunity to develop before the other bacteria were sown. It is not surprising, then, that there was an increase for the first few days. At the end of the month, however, there was a very marked antagonism, the decrease amounting to practically 100 per cent. in the first two and last cases, and 78 and 47 per cent. in the other two cases respectively.

In the third and fourth cases it is to be noticed that the conditions are different from those in the first two cases. Here the opposing bacteria were given a distinct start, and the number of determinations were fewer and extend only over a week's time, and not a month as in the preceding cases.

SERIES VII. WATER BACTERIA.

There being an antagonism distinctly observable when broth is seeded with the bacteria from water, it was decided to determine whether the antagonistic substances are developed when grown in water instead of broth. Flasks were, therefore, fitted up with collodion sacs and filled with lake water and sterilized in the autoclave, and the sacs were seeded with *B. typhosus*, and the water in one of the flasks was inoculated with water bacteria by adding a few drops of raw lake water. The other flask was not inoculated.

The culture used was the M. H.

The temperature was that of the room in summer, 20-22°.

The results are indicated in the following Table VII:

TABLE VII.
EFFECT OF STERILE AND RAW WATER ON THE B. TYPHOSUS.

No.	SOURCE OF ORGANISMS		PERIOD OF PRELIM. CULTIVATION		TEMPERATURE	NO. OF COLONIES OF B. TYPHOSUS DEVELOPING ON AGAR FROM ONE LOOP OF THE MEDIUM						RE-SULT +Inc. -Dec.
	Opp.	B. ty.	Opp.	B. ty.		0	1 D.	2 D.	4 D.	6 D.	30 D.	
1.....	Sterile water	M. H.	0	0	20-22° C	4,400	1,600	940	590	270	0	-100%
2.....	Raw water	M. H.	0	0	20-22° C	2,900	590	380	30,000	30.00	Very few	-100%

There is in sterile water a gradual, but distinct, decrease, which in a relatively short time amounts to extinction.

In the raw water the decline was checked at the end of the second day, and the number began to increase and was maintained for some days, and then began to decline again; but at the end of one month's time *B. typhosus* had not entirely disappeared. During this time, however, the number of water bacteria in the flask outside of the sac had increased to an enormous extent, as they always do under similar circumstances, *i. e.*, when water is inclosed in a small space and kept at a temperature which will permit of the growth of bacteria. It is true, then, that the water in the flask after the lapse of a few days is a very different water from what it was when *B. typhosus* was first introduced.

SERIES VIII. WATER BACTERIA.

In view of the results obtained in the previous series, it was decided to try to grow *B. typhosus* in a collodion sac in water kept under conditions which would keep down its germ content; in other words, to grow *B. typhosus* in the lake. For various reasons the sacs were not put in the lake itself, but were kept in the laboratory sink, under conditions, to be presently explained, which were, so far as can be seen at the present time, exactly similar in effect to those which obtained in the lake.

This procedure was intended to keep the water in motion, and thus prevent the enormous multiplication of the germs which always occurs when water taken from a large body of water is confined in a small vessel. For this purpose an apparatus was arranged as indicated in Fig. 6.

The collodion sacs were made as usual and sterilized in flasks of water. The sacs with their sterile water content were then placed in the running-water, where they were left without inoculation for twenty-four hours. Cultures of the water in the sacs were then made in order to determine whether the sacs were tight or not. In some cases it was found that they were not

tight, and in these cases they were replaced with new ones, which were in turn tested in the same way. When the sacs were found to have remained in running water for twenty-four hours, without allowing the water bacteria to gain entrance, they were inoculated with *B. typhosus*. Cultures were made at this

time and at regular periods for twenty days.

The experiment was conducted in duplicate, as will be seen, and was also accompanied by a control in sterile water, which was also conducted in duplicate. The culture was the M. H.

The temperature of the water, of course, varied. A thermometer was kept in the apparatus all of the time, and the temperature was read each morning and recorded.

The results are shown in Table VIII.

In the case of the controls, or boiled water, there was a decline in the first day or two, which was followed by a marked and very sudden increase, and then a gradual decline, which at the end of twelve days amounted to extinction, so far as the amount tested was concerned.

In the raw, or running, water there was apparently no decline, but in one of the two cases there was an in-

crease observed in the first few days, and then a decline which reached a low point in seven days, or five days sooner than in the case of boiled water. It must be noted, however, that *B. typhosus* persisted quite as long in this case as in the boiled water; indeed, these figures seem to indicate that at the end of twenty days they were more numerous, per loop of material, than were the typhoid germs at the same time in the boiled water.

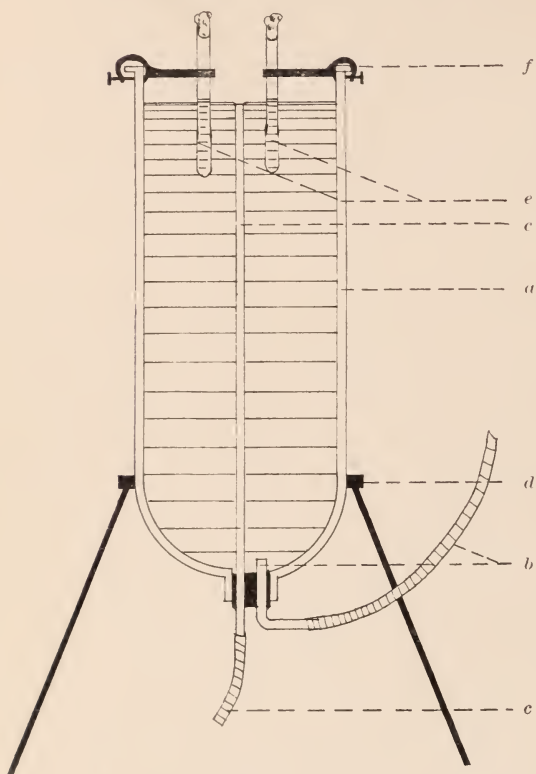


FIG. 6.—Apparatus for immersing collodion sacs in running water. *a*, glass bell jar; *b*, inlet tube; *c*, outlet tube; *d*, tripod; *e*, collodion sacs sealed on test-tubes and inoculated with the typhoid germ, held in place by the iron clamps *f*.

TABLE VIII.

EFFECT OF STERILE AND RAW LAKE WATER ON THE *B. TYPHOSUS*.

NO. OF EX-PER-IMENT	OPPOSING MEDIUM	CUL-TURE OF <i>B. TYPHOSUS</i> USED	TEMPERATURE	NUMBER OF COLONIES OF <i>B. TYPHOSUS</i> DEVELOPING ON AGAR FROM ONE LOOP OF MEDIUM									RE-SULT + Jn. - De.
				0	1 D.	2 D.	5 D.	7 D.	11 D.	12 D.	16 D.	20 D.	
1	Boiled water	M. H.	18-23	600	360	4,750	400	100	6	2	0	0	-100%
2	Boiled water	M. H.	18-23	450	125	325	6,500	400	600	15	1	1	-100%
3	Raw water	M. H.	18-23	300	300	480	200	10	5	- 98%
4	Raw water	M. H.	18-23	200	175	135	85	16	16	10	10	10	- 95%

No very definite conclusions seem warranted from these results. It appears, however, that there were conditions in the boiled water which permitted a rapid and extensive development for a few days. There seems to have been no shortening of the life of *B. typhosus* in raw, or running water. If the water bacteria exert a prejudicial effect on *B. typhosus*, either it is so slight that it cannot be detected by this means, or it does not pass through the collodion sac. This result is not in accord with numerous investigators on this subject.

GENERAL DISCUSSION OF RESULTS.

The results of the experiments already recorded show that there is a marked antagonism exerted by soil and water bacteria on *B. typhosus* when the same are grown in broth and *B. typhosus* are immersed therein, for, whereas *B. typhosus* in the collodion sac in sterile broth grows rapidly and maintains itself in enormous numbers for long periods of time, it rarely multiplies to any extent in the broth saturated with the by-products of these bacteria, and invariably shows, usually in a few days, a rapid decline, which in the course of a few days, or weeks at most, amounts to practical extinction in the quantities tested.

The antagonistic effects are produced more rapidly, and the effect is more marked, when the opposing organisms are allowed to develop their by-products before *B. typhosus* is introduced; and, *vice versa*, where *B. typhosus* is allowed to develop before

the opposing germs are introduced, some considerable time is required to demonstrate the antagonism, and the action is slower and less marked. The time required to develop the antagonistic by-products is comparatively short, very positive results being obtained in forty-eight to seventy-two hours. The influence which the prolongation of the period of incubation of the opposing bacteria has on the rapidity of the extinction of *B. typhosus* is well shown in the following table. The *B. typhosus* was grown in the collodion sac, as in the preceding experiments, and the opposing organisms were from soil No. 3. The temperature was 28°, and the culture of *B. typhosus* was the M. H.

TABLE IX.

THE EFFECT OF PROLONGING THE PERIOD OF INCUBATION OF THE OPPOSING BACTERIA ON RATE OF EXTINCTION OF *B. TYPHOSUS*.

NO. OF EXPER- IMENT	DAYS OF PRELIMINARY CULTIVATION		DAY BEFORE EX- TINCTION	PER CENT. DECREASE
	<i>B. typhosus</i>	Opposing Bacteria		
1	0	12	2	100
2	0	6	5	100
3	2	0	7	100

A question of considerable importance is this: Do soil and water bacteria growing in their natural habitats produce by-products in sufficient quantities to saturate these substances, and thus give to them a germicidal property? This has been very frequently tested for the water bacteria, and while there seems to be no consensus of opinion as to the germicidal properties of the natural waters, there is abundant proof that raw waters are not suited for the development of *B. typhosus*, but the heating of the same in the process of sterilization renders the water capable of sustaining the organism for a longer time, and under some conditions permits the multiplication of the typhoid germ in them. This point does not seem to have been determined for soils. The following experiment was made in this connection: Soil was collected from a plowed field. The bacteria in this soil had already been shown to possess antagonistic properties, since it

was the same as soil No. 6 in Series V. One kilogram of this soil was placed in a large funnel, and to it one liter of boiled tap water was added. The first 300 c.c. were caught and filtered through a Pasteur-Chamberland filter (No. F) and distributed into two small, sterile flasks. One of these was afterward run through an autoclave. A similar flask of autoclaved tap water was used as a control. These flasks were heavily seeded with *B. typhosus* (P. D. 1.) and put at a temperature of 38° , so that if there were no antagonistic substances present, *B. typhosus* would probably multiply. The results obtained follow:

TABLE X.

CAREER OF *B. TYPHOSUS* EXPOSED TO RAW, FILTERED SOIL LEACHINGS; BOILED, FILTERED LEACHINGS; AND BOILED TAP WATER.

NO. OF EXPERI- MENT	DESCRIPTION	NO. TYPHOID COLONIES FROM TWO LOOPS		
		0	2 Days	3 Days
1	Filtered leachings.....	500	12,000	35,000
2	Filtered leachings (autoclaved)	500	20,000	20,000
3	Tap water (autoclaved).....	2,000	70	17

From the data recorded in the above table there seems to be no evidence whatever that there are any substances present in the soil which are antagonistic for the typhoid germ. There is apparently no difference between the raw and autoclaved leachings, and at 38° there is a marked increase in the number of germs in three days. This is in strong contrast with the condition of affairs in the case of the autoclaved tap water, where under identical conditions there is a marked decrease. This difference is probably to be explained by the presence of a large amount of organic matter in the soil leachings which furnishes conditions favorable for the rapid development of *B. typhosus*. It seems probable, then, that the antagonism which a soil may possess for *B. typhosus* is due to the bacteria present and the by-products which they may produce in the immediate presence of the typhoid germ, and not to by-products previously formed.

The bacteria which are antagonistic to *B. typhosus* seem to

be widely distributed in the soil and water. So far, they have been found in practically all of the samples examined. These include soils from various sources, as woods, grass plots, plowed ground, road dust, clay from several feet below the surface, and sand, as well as various waters.

ANTAGONISM EXHIBITED BY PURE CULTURES OF CERTAIN BACTERIA.

In the previous section the fact is shown that when *B. typhosus* is grown on one side of a permeable membrane, as a collodion film, and a mixed culture of soil or water bacteria on the other side, *B. typhosus* is not only unable to grow, but in most cases rapidly dies out. The question then naturally arose whether it was possible to secure single species of bacteria which, when grown in pure culture, would produce a similar result. With this end in view the following experiment was performed:

After Series II (p. 610) had been running for eight days, a number of agar plates were made from each flask in order to isolate the various bacteria present for further study. But as there was an interruption of the work at this time, the plates remained in the ice-chest for about two months. Subcultures were then made from the various colonies, and after they had grown for a few days on agar slopes they were inoculated into flasks fitted with collodion sacs. The sacs had been inoculated with *B. typhosus* twenty-four hours earlier. The stock of *B. typhosus* was P. D. 1. The temperature of the experiment was 28°. Cultures from seven different colonies were tested in this way. In regard to the results it may be stated, without going into greater detail, that there was no decline in the numbers, as was expected, but that there was in all cases a four, or more, fold increase and that these numbers were maintained for at least one month. These results may be accounted for on any of the following suppositions: First, it is possible that the particular organism possessing antagonistic properties was not obtained in the plate cultures. But it is to be remembered that the plates were made when the antagonistic properties were very strong, and it would be expected that this organism would be more or less

abundant in the mixture. Second, it is possible that the organisms, although normally antagonistic, may have lost their peculiar properties through the long standing in the plate culture. Third, it may also be true that the antagonism exerted in the former experiments is due, not to a single organism, but the combined action of two or more different species.

While the last experiment was in progress, and as a check on the same, it was determined to test the effect of growing pure cultures of certain bacteria in close proximity to *B. typhosus* in solid media, somewhat after the method used by Garré. (Method 1, p. 602.) Therefore a series of agar plates were made which were heavily seeded with *B. typhosus*. After the agar had thoroughly solidified, streaks were made on the surface of the agar with the organisms to be tested. These were new cultures from soil No. 4, the same as that used in Series II, a sample of which had been kept. After twenty-four hours at 28° it was found that the bacteria growing in the streaks on the surface of the media had in one case seriously interfered with the growth of *B. typhosus* in the agar immediately beneath. In this case *B. typhosus* had not developed into visible colonies beneath the streak and for some distance on each side of the streak.

By this means an organism was discovered which showed strong antagonistic properties. This culture proved to be, on further study, the "potato bacillus," or *B. vulgaris* Trevisan.

A number of pure cultures were now tried in the same way. These included the following well-known bacteria: *Pseudomonas fluorescens* (Fluegge) Migula (*B. fluor. liquefaciens*); *Pseudomonas aeruginosa* (Schroeter) Migula (*B. pyocyaneus*); *Pseudomonas putida* (Fluegge) Migula (*B. fluorescens putidus*); *Bacillus prodigiosus* (Ehrenberg) Fluegge; *Bacillus vulgaris* (Hauser) Migula (*Proteus vulgaris*).

In this way an antagonism was detected for *B. typhosus* in the case of three out of five germs, viz.: *Ps. fluorescens*, *Ps. putida*, and *B. vulgaris*. A number of experiments with these three germs, together with *B. vulgaris*, were carried out, and the antagonism which they exert on *B. typhosus* was very definitely shown.

Two courses were now open: the various bacteria in the soils already examined could be isolated, and the relation of each individual species to *B. typhosus* could be determined and their distribution studied; or the organisms already found could be studied for the purpose of determining the extent of the antagonism, the influence of various factors in modifying the antagonism, and finally the cause of the antagonism could be investigated. The latter course was determined on, and it is now proposed to discuss the results in detail.

ANTAGONISM EXHIBITED BY *B. VULGATUS* TREVISAN.

This organism is usually designated, incorrectly, by the trinomial, *Bacillus mesentericus vulgatus* Fluegge. The common name is the "potato bacillus."

The culture used was isolated from soil No. 4 in Series II (p. 611), and at first designated by number as 59-4, but upon further study it has proved to be *B. vulgatus*.

No reference in literature has been found to the antagonistic action of this germ for *B. typhosus*, but, as the following experiments show, it possesses a strong antagonistic action.

Agar plates were heavily seeded with *B. typhosus*, and when the agar was hard, it was streaked with *B. vulgatus*. The results follow:

TABLE XI.
ANTAGONISM EXERTED BY *B. VULGATUS*.

No. of Exp'm't	Temperature	Kind of Medium	Results
1	28	Nutrient agar	Antagonism apparent.
2	28	"	Marked antagonism.
3	28	"	Strongly marked ($\frac{1}{4}$ inch clear agar around streak).
4	28	Hiss's medium	Strongly marked antagonism.

The presence of an antagonism was also shown by means of the double-plate method (No. 6, p. 604) where *B. typhosus* repeatedly failed to grow on the side of the plate seeded with *B. vulgatus* (see Plate XVIII, Fig. 1, A).

Again, the agar-block method was used. The *B. typhosus* was inoculated into agar blocks, and these were then put into broth cultures of the potato bacillus. Repeated trials have shown that under these conditions the typhoid germ invariably fails to grow. (See Plate XVIII, Fig. 2, D.)

This series of experiments shows clearly the antagonism which is exerted, since in all cases controls were made in which *B. typhosus* was grown on itself.

TABLE XII.

INFLUENCE OF PRELIMINARY CULTIVATION OF *B. vulgatus* ON
DEVELOPMENT OF ANTAGONISM.

No. of Experiment	Temperature	Time Allowed <i>B. vulgatus</i> to Grow before <i>B. typhosus</i> Is Seeded	Results
1	28	0 hours	No antagonism.
2	28	0 "	Slight (if any).
3	28	6 "	Slight antagonism.
4	28	48 "	Marked antagonism.

The above table brings out another fact, and that is that it requires some time for *B. vulgatus* to develop its antagonistic products. Thus it happens that, if *B. typhosus* is seeded at the time that the other germ is seeded, it has time to develop into a visible streak before its growth is checked. This happened in Nos. 1 and 2. When, however, *B. vulgatus* is given a start, it invariably prevents the development of *B. typhosus*.

The antagonism is also demonstrable when this germ is grown in broth and *B. typhosus* is immersed in a collodion sac therein, as shown in the following table. The temperature of the experiment was 28°, and the culture of *B. typhosus* was the P. D. 2.

TABLE XIII.

ANTAGONISM OF *B. vulgatus*.
(Collodion Sac Method.)

PERIOD OF PRELIMINARY CULTIVATION		NO. OF COLONIES OF <i>B. typhosus</i> PER LOOP						
<i>B. vulgatus</i>	<i>B. typhosus</i>	0	1 Day	2 Days	3 Days	4 Days	5 Days	6 Days
12 days	2 days	1,400	360	40

ANTAGONISM EXHIBITED BY *BACILLUS VULGARIS* (HAUSER) MIGULA.

This organism is usually known as *Proteus vulgaris*;* occasionally as *Bacillus proteus*.†

There seems to have been no experimental work on the antagonism exerted by *B. vulgaris* for *B. typhosus* recorded, although the idea is quite general that the group of organisms to which this belongs, *i. e.*, the *Proteus* group, does possess antagonistic properties for the typhoid germ. For instance, Sidney Martin,⁵ in giving the reasons for the disappearance of the typhoid

* HÄUSER, *Ueber Fäulnisbakterien*, 1885.

† TREVISAN, *Genera*, 1889.

germ from unsterilized, virgin soil, remarks that the disappearance seems to be due to the antagonism of the soil micro-organisms, and stated that "there is some evidence to show that, under the conditions of the experiment, the disappearance of the bacillus [*B. typhosus*] has taken place *pari passu* with the increase of the number of the putrefactive bacteria."

The source of the particular culture used was the Laboratory of the Johns Hopkins Hospital, where it was known as 22*b*. This germ was isolated from several of the soils used.

Experimental work.—Agar plates were heavily seeded (Method 1, p. 602) with *B. typhosus*, and when the medium had hardened the surface was streaked with *B. vulgaris*. The plates were incubated at 38°. Twenty-four hours later it was invariably found that there was a marked antagonism for *B. typhosus*. Usually the colonies were small under the streak and for some distance each side of the streak. (See Fig. 2.) In some cases the typhoid colonies under the streak of *B. vulgaris* did not develop at all, and the agar remained clear for a short distance each side of the streak. The results obtained in this series of experiments is shown in the following table:

TABLE XIV.
ANTAGONISM OF *B. VULGARIS*.

No. of Experiment	Temperature	Kind of Medium	Results
1	38	Nutrient agar	Marked antagonism
2	38	" "	" "
3	36	" "	" "
4	38	" "	" "
5	38	" "	" "

The antagonism was also tested by the double-plate method, and the results obtained are shown in the following table (see also Fig. 5, *B*):

TABLE XV.
ANTAGONISM OF *B. VULGARIS* TESTED BY THE DOUBLE-PLATE METHOD.

No. of Experiment	Temperature	Medium	Time Allowed <i>B. vulgaris</i> before seeding <i>B. typhosus</i>	Results
1	28	Nutrient agar	0 hours	Marked
2	28	" "	0 "	"
3	28	" "	6 "	"
4	28	" "	6 "	"
5	28	" "	48 "	Strong

It will be noticed here, in contrast to the condition of affairs under *B. vulgatus*, that *B. vulgaris* develops its antagonistic properties immediately and, in the experiments performed here, there was no case where *B. typhosus* was able to grow on the seeded half of the plate.

This organism has been grown in broth, and in these cultures agar blocks have been immersed, and it has been shown repeatedly that *B. typhosus* will not develop in the by-products of this germ. (See Plate XVIII, Fig. 2, *E.*)

The antagonism is also demonstrable when this germ is grown in broth and *B. typhosus* is immersed in a collodion sac therein, as shown in the following table. The temperature of the experiment was 28° and the culture of *B. typhosus* was the P. D. 2.

TABLE XVI.
ANTAGONISM OF *B. VULGARIS*.
(Collodion Sac Method.)

PERIOD OF PRELIMINARY CULTIVATION		NO. OF COLONIES OF <i>B. TYPHOSUS</i> PER LOOP						
<i>B. vulgaris</i>	<i>B. typhosus</i>	0	1 Day	2 Days	3 Days	4 Days	5 Days	6 Days
12 days	2 days	3,200	3,600	1,500

ANTAGONISM EXHIBITED BY THE *PSEUDOMONAS FLUORESCENS*
(FLUEGGE) MIG.

This germ is usually spoken of as the *Bacillus fluorescens liquefaciens*.^{*} It has also been described as *B. viscosus*[†] and *B. Fluorescens nivalis*.[‡]

The particular cultures used here were one isolated from the water in Lake Mendota and one obtained from Professor Jordan, of the University of Chicago.

The antagonism exhibited by *Ps. fluorescens* for *B. typhosus* was apparently first described by Olitzky.³ This investigator found that the organism in question exerted a strong restraining influence on *B. typhosus*, and suggested that this fact might have practical hygienic importance. The methods used by this worker were slight modifications of Garré's methods.

Laws and Andrews,⁴ working on the duration of the life of *B. typhosus* in sewage, tested the effect of the presence of different organisms on *B. typhosus*, and among them *Ps. fluorescens*, and concluded that it had no effect on *B. typhosus*.

* FLUEGGE, *Mikroorganismen*, 1886, p. 289.

† FRANKLAND, *Ztschr. f. Hyg.*, 1887, 6, p. 39.

‡ SCHMELK, *Centralbl. f. Bakt.*, 1888, 4, p. 514.

Horrocks,⁷ working on similar lines, found that he could not isolate *B. typhosus* from sewage after seven days, when *Ps. fluorescens* was present, but could isolate it from sterile sewage two months after inoculation. He did not, however, feel sure but that some typhoid colonies had escaped him in the first case. He also found that *B. typhosus* would not grow on the surface of gelatin which had already yielded a growth of *Ps. fluorescens*.

The effect of this organism on *B. typhosus* has been reinvestigated by means of the new methods already described.

Agar plates were seeded with *B. typhosus*, and when the agar was well hardened *Ps. fluorescens* was streaked over the surface. In most cases it was found upon development that the colonies under and immediately around the streak of *Ps. fluorescens* had either not grown or were much smaller than in other parts of the plate. The following table gives the data in detail:

TABLE XVII.

ANTAGONISM EXERTED BY THE *PSEUDOMONAS FLUORESCENS* DEVELOPING ON AN AGAR TYPHOID PLATE.

No. of Experiment	Temperature	Medium	Result
1	28°	Nutrient agar	Antagonism apparent
2	28	" "	" "
3	28	Sugar-free agar	" "
4	28	" "	" "

From these experiments it seems fair to conclude that when the two organisms are grown as in the preceding experiments there is a marked inhibiting effect exerted by *Ps. fluorescens* on *B. typhosus* which frequently extends through several millimeters of agar.

The difficulty which *B. typhosus* has in growing in the presence of *Ps. fluorescens* is further shown in the following experiments with the double-plate method (see Plate XVIII, Fig. 1, C):

TABLE XVIII.

ANTAGONISM OF *PS. FLUORESCENS* TESTED BY DOUBLE-PLATE METHOD.

No. of Experiment	Temperature	Time Allowed Antibiotic to Develop before Seeding <i>B. typhosus</i>	Result
1	28	0 hours	Very slight antagonism
2	28	0 "	Slight antagonism
3	28	6 "	Marked antagonism
4	28	48 "	Very marked antag.

In addition to the fact that there is an antagonism, the further fact is brought out that time is required for the development of the maximum amount of antagonism.

The antagonism has been further tested by means of the agar-block method, and in no case was *B. typhosus* able to grow in the by-products of this germ (see Plate I, Fig. 2, *F*).

The antagonism is also demonstrable when this germ is grown in broth and *B. typhosus* is immersed in a collodion sac therein, as shown in the following table. The temperature of the experiment was 28° and the culture of *B. typhosus* was the P. D. 2.

TABLE XIX.
ANTAGONISM OF *Ps. fluorescens*.
(Collodion Sac Method.)

PERIOD OF PRELIM. CULTIVAT'N		NUMBER OF COLONIES OF <i>B. TYPHOSUS</i> PER LOOP						
<i>Ps. fluorescens</i>	<i>B. typhosus</i>	0	1 Day	2 Days	3 Days	4 Days	5 Days	6 Days
30 days	0	720	0	0

ANTAGONISM EXHIBITED BY *PSEUDOMONAS PUTIDA* (FLUEGGE)
MIGULA.

This organism is usually known as the *Bacillus fluorescens putidus* Fluegge, and differs slightly, if at all, from the *Bacterium immobile* (*B. fluorescens non-liquefaciens*) except in motility.

The culture used here was one obtained from Professor Jordan, of the University of Chicago.

Garré² has called attention to an antagonism exerted by *Ps. putida* on the *B. typhosus*, but apparently no one else has studied it.

The antagonism has been studied by means of the double-plate method and found to be marked, although apparently not as strong as in the case of *Pseudomonas fluorescens* (see Plate I, Fig. 1, *D*).

In the agar blocks there is usually no growth along the streak, although occasionally a slight growth does occur as in the tube represented in Plate I, Fig. 1, *G*.

An antagonism is demonstrable by means of the collodion-sac method, as the following table will show. The temperature of this experiment was 28°, and the culture of *B. typhosus* used was P. D. 2.

TABLE XX.
ANTAGONISM OF *Ps. putida*.
(Collodion Sac Method.)

PERIOD OF PRELIM. CULTIVAT'N		NUMBER OF COLONIES <i>B. typhosus</i> PER LOOP						
<i>Ps. putida</i>	<i>B. typhosus</i>	0	1 Day	2 Days	3 Days	4 Days	5 Days	6 Days
12 days	2 days	1,200	...	650	150

GENERAL DISCUSSION OF RESULTS.

These organisms all show a marked antagonism for *B. typhosus* when tested as above described. Of the four *Pseudomonas fluorescens* exhibits the strongest antagonistic properties, although it requires some time to develop this properly. *B. vulgaris* acts more promptly than the others, but the antagonistic substance does not diffuse to so great a distance in the medium.

These germs are among the most common and widely distributed of the soil and water bacteria.

These germs all produce substances which pass through a collodion film. The results obtained here by this method, however, have not been as striking in case of *B. vulgatus* and *B. vulgaris* as in the case of the mixed cultures; partly, no doubt, because the cultures had been grown for a longer time on artificial culture media; and, furthermore, it is quite likely that the greater antagonism in the mixed cultures may have been due to the combined action of two or more species.

EFFECT OF VARIOUS AGENTS IN INFLUENCING ANTAGONISM.

A series of experiments were undertaken to determine the effect of various agents in influencing the antagonism exerted by these germs.

INFLUENCE OF INCUBATING TEMPERATURE ON THE DEVELOPMENT OF ANTAGONISM.

Double plates were made and one-half seeded with these organisms, and then a few hours later both sides were streaked with *B. typhosus*. The plates were kept at the temperature of the ice-chest ($10-12^{\circ}$), room temperature ($17-20^{\circ}$), low incubator (28°), and blood heat (38°), with the following results:

TABLE XXI.

EFFECT OF INCUBATION TEMPERATURE ON ANTAGONISM.

ORGANISM	TEMPERATURE			
	10-12	17-22	28°	38°
<i>B. vulgatus</i>	0	0	0	0
<i>B. vulgaris</i>	0	0	0	0
<i>Ps. fluorescens</i>	0	0	0	0
<i>Ps. putida</i>	0	0	0	0

0 means no growth.

The effect of incubation temperature was tested in another way, as follows:

Tubes of neutral broth were inoculated with the four organisms and grown at the different temperatures. These were allowed to grow six days, and at the end of that time there was a good growth even in those tubes kept at the ice-chest temperature. All of these tubes were then heated to 60° for ten minutes.

An agar block seeded with *B. typhosus* was then placed in each tube, and all placed at the temperature of 38°.

The purpose of the heating was to kill the vegetative forms and thus prevent, as much as possible, the formation of the antagonistic substance at 38°. The heating had been found not to interfere with the action of the antagonistic substance already formed. Twenty-four hours later the following results were obtained:

TABLE XXII.

EFFECT OF INCUBATION TEMPERATURE ON ANTAGONISM.

ORGANISM	TEMPERATURE			
	10-12	17-22	28°	38°
<i>B. vulgatus</i>	+	+	0	0
<i>B. vulgaris</i>	0	0	0	0
<i>Ps. fluorescens</i>	+	+	0	0
<i>Ps. putida</i>	++	+	Contaminated; had a pellicle	+

+ means that the streak was visible.

++ means that the streak was distinctly visible.

0 means that the streak was not visible.

From these results it would appear that the temperature variations do not have any appreciable effect, as measured by the means at command; that the temperatures which permit growth also permit the production of the antagonistic substances.

ACTIVITY OF ANTAGONISTIC SUBSTANCES AT DIFFERENT TEMPERATURES.

The germs here were allowed to grow under as nearly as possible optimum conditions, and when they had had the opportunity to develop their by-products, the broth cultures were divided and a portion of each put at the different temperatures. At this time an agar block, seeded with the typhoid germ, was placed in each, and they were allowed to remain six days at the different temperatures. The results were as follows:

TABLE XXIII.

ACTIVITY OF ANTAGONISTIC SUBSTANCES AT DIFFERENT TEMPERATURES.

ORGANISM	TEMPERATURES			
	10-12	17-22°	28°	38°
<i>B. vulgatus</i>	++	++	+	0
<i>B. vulgaris</i>	++	++	0	0
<i>Ps. fluorescens</i>	++	++	0	0
<i>Ps. putida</i>	++	++	+	0

++ means slight growth.

+ means that the streak was visible.

0 means that the streak was not visible.

This indicates that the action of these antagonistic substances varies somewhat with the temperatures, that at high temperatures—that of the body—the action is most pronounced, and that at the temperature of the ice-chest it is so delayed that *B. typhosus* has an opportunity to develop. This appears to be a point of great interest, and offers at least a partial explanation of the singular fact that water supplies which become contaminated in cold weather retain their power of producing infection for a much longer time than when the contamination occurs in warm weather.

HEAT STABILITY OF ANTAGONISTIC SUBSTANCES.

The attempt to determine the temperature which was destructive to these substances was made as follows:

A large flask of broth was seeded with the *Ps. fluorescens* and kept at 28° for about two weeks. From this flask a number of sterile test-tubes were filled and heated at various temperatures—50°, 55°, 60°, 65°, 70°, 75°, 80°, and 100°—for ten minutes. These tubes then had placed in each an agar block seeded with *B. typhosus*. They then were incubated at 38°. The typhoid germ failed to develop in any, although it grew well in a tube of sterile broth used as a control.

Agar tubes were then melted, cooled, seeded with *Ps. fluorescens*, and allowed to grow as shake cultures for a week. They were then heated in the autoclave at 120° for ten minutes. The melted culture was then allowed to solidify in a sloping position, and later streaks of *B. typhosus* were made and the cultures incubated at 38°. The typhoid streak did not develop, showing that the restraining substance had not been destroyed.

The above experiments show that these antagonistic substances are thermo-stable, being able to withstand the action of 120° C. for ten minutes at least; and in this connection it is interesting to note that Abbott and Gildersleeve¹⁶ have recently shown that the by-products of some of the common bacteria may be heated to 100° C. for fifteen to thirty minutes without changing their hemolytic activities.

INFLUENCE OF DEXTROSE IN THE MEDIUM.

Double plates were made, and the organism whose antagonistic properties was to be tested was seeded on one half in ordinary nutrient media, made from extract of beef, and therefore, practically sugar free. In some cases sugar-free agar was used, made according to Smith's well-known directions. On the other half the organism was sown in agar containing 1 per cent. of dextrose. A few hours later the plates were streaked with *B. typhosus* and incubated at 28°.

No very definite results could be obtained with any of the above organisms, although an organism that did not antagonize the typhoid germ when grown on sugar-free media was strongly antagonistic on the dextrose media.

A further attempt was made to study this with agar blocks, but it was likewise impossible to determine any influence exerted by the presence of dextrose.

INFLUENCE OF OXYGEN ON DEVELOPMENT OF ANTAGONISTIC SUBSTANCES.

Double plates were made, and one set kept as usual in the air, and another in a Novy jar from which the oxygen was absorbed by the use of an alkaline pyrogallie acid solution. Growth in the absence of oxygen did not seem to affect in any way the development of the antagonistic substances, which were apparently as strong under one condition as the other.

INFLUENCE OF REACTION OF MEDIUM ON THE DEVELOPMENT OF ANTAGONISM.

To test this the different bacteria were grown in broth to which varying amounts of normal acid and alkali had been added. The amount is indicated in the Fuller scale. After the cultures had been incubated several days, agar blocks seeded with *B. typhosus* were added, and the following results obtained :

TABLE XXIV.
INFLUENCE OF REACTION OF MEDIUM ON ANTAGONISM.

Reaction of Medium Expressed in Per Cent. Normal Solutions	+1.5	0	-1.5	-3	-5
<i>B. vulgatus</i>	—	0	0	—	—
<i>B. vulgaris</i>	—	0	0	—	—
<i>Ps. fluorescens</i>	—	0	0	0	0
<i>Ps. putida</i>	—	+	+	+	—

— means no growth of bacterium and therefore agar block not added.

0 means streak was not visible.

— means that streak was visible, but no growth.

These results would indicate that the reaction of the culture medium was not effective in influencing the production or action of the antagonistic substances.

GENERAL DISCUSSION OF RESULTS.

In reviewing the behavior of these organisms under the conditions studied above, it would seem that the various agents have little, if anything, to do in influencing the development of the antagonism which they exhibit for *B. typhosus*.

Conditions which favor the growth of the various bacteria undoubtedly favor the development of the antagonistic properties,

in that they hasten their production, but there is no evidence at hand to show that these substances are not produced whenever a good growth occurs.

While, then, the antagonistic properties are produced in all well-developed cultures, they do not seem to be equally active under all conditions, and especial attention is called to the fact, already developed, that at low temperatures the antagonism exhibited is appreciably less than it is at higher temperatures—a fact apparently of great hygienic significance.

POSSIBLE EXPLANATIONS OF ANTAGONISM.

THEORY OF EXHAUSTION OF FOOD SUPPLY.

One of the first theories advanced to explain the antagonism exerted by one bacterium for another was that it was due to an exhaustion of the food supply. Olitzky³ controverted this and presented the evidence that *Micrococcus aureus* would grow on media which had supported a previous crop of bacteria, but which would not permit the growth of *B. typhosus*. The following experiment also clearly demonstrates that the food-exhaustion theory is not a sufficient explanation. *Ps. fluorescens* was grown in broth for some days, and then the culture was pasteurized, and part of it was placed in a sterile test-tube, and part in an equal quantity of sterile broth. To each test-tube was then added an agar block of *B. typhosus*. *B. typhosus* failed to grow in either, although it grew well in a control tube of sterile broth, thus showing that growth is prevented in the presence of abundant food material.

THEORY OF ENZYME ACTION.

The theory that the antagonistic action is due to an enzyme is one that is frequently held. This, of course, could not be the explanation in all cases, because organisms, as *Ps. putida*, which do not produce enzymes, exert an antagonistic action. In the case of liquefiers, however, it might be explained in this way.

Houston,¹⁷ in discussing the survival of pathogenic organisms after passing through bacterial filter beds, says:

.thirdly, that the balance of evidence points to the probability that some, at all events, of the pathogenic organisms are crowded out in the

struggle for existence in a nutritive medium containing a mixed bacterial flora, their vitality being weakened or destroyed by the enzymes of Saprophytic Species.

It seems very doubtful if this antagonism is due to enzymes.

The commercial enzymes, in powdered form were tested on *B. typhosus* by means of the double-plate method, and in no case was there a definite antagonistic action observed. The enzymes tested were Taka diastase, pepsin, trypsin, and pancreatin.

Nor would it be possible to explain the antagonistic action observed in the case of the collodion sacs on this theory, since the enzymes are colloidal in nature, and they do not pass through the collodion film, as the following experiment shows:

A collodion sac was prepared in the usual way, and sterilized in a flask of gelatin instead of broth, so that when the gelatin had hardened the sac was imbedded in it. The sac was then inoculated with a liquefying organism (*Pseudomonas aeruginosa*) and allowed to stand for some time. This finally amounted to about six months. During this time there was abundant growth in the sac, but no effect on the gelatin outside. To show that the culture had not lost its power to produce an enzyme, or had not become otherwise modified, some of the culture was introduced into an ordinary gelatin tube, where it produced the ordinary, characteristic changes, and then the gelatin outside of the sac was inoculated by rupturing the sac, when there took place the rapid liquefaction of the gelatin in the flask. It, therefore, seems quite certain that the enzymes cannot pass through the collodion sac, and so are not capable of explaining the action.

It is further shown by the following experiment that the antagonistic action in the case of *Ps. fluorescens*, at least, cannot be due to the enzymes produced.

It will be recalled that this substance is thermo-stable, but it can be readily shown that the proteolytic action of the products of this germ, as tested by means of milk-agar blocks, is largely, if not completely,* destroyed by an exposure to 75° C. for ten minutes. This seems sufficient proof that the substance is not identical with the proteolytic enzyme produced by this organism.

* In this connection it is worthy of note that these results do not agree with those of Abbott and Gildersleeve.¹⁶

THEORY OF SPECIFIC POISONS.

Newman,¹⁸ in his book on bacteria, says:

In several of the most recent of the admirable reports of Sir Richard Thorne, issued from the medical department of the Local Government Board, we have the record of a series of experiments performed by Dr. Klein into this question of the antagonism of microbes. From this work it is clearly demonstrated that whatever opposition one species affords to another it is able to exercise by means of its poisonous properties. These are of two kinds. There is, as is now widely known, the poisonous product named the toxin. There is also in many species, as Dr. Klein has pointed out, a poisonous constituent or constituents included in the body protoplasm of the bacillus, and which he therefore terms the *intracellular poison*. Now, whilst the former is different in every species, the latter may be a property common to several species. Hence those having a similar intracellular poison are antagonistic to each other, each member of such a group being unable to live in an environment of its own intracellular poison.*

While this explanation is an attractive one, it does not seem to satisfy the conditions. In the first place, if the poisonous substances were intracellular, one would expect them to be colloidal. These are not, since they diffuse through the collodium film. Again, these intracellular poisons are probably of the nature of proteid compounds and thermo-labile. The poisonous substances here have been found to be thermo-stable. And, finally, if the antagonism is due to similar intracellular poisons, one would expect that the poison of the typhoid germs would be quite as antagonistic for itself as other intracellular poisons are for it. This is not true. The typhoid germ will grow on itself, to a somewhat limited extent, to be sure, but in striking contrast to the condition of affairs in the other cases.

THEORY OF ACID AND ALKALI PRODUCTION.

It has further been suggested that the antagonistic action is due to changes in the reaction of the culture medium. Sirotinin¹⁹ suggested this, and claimed that by neutralizing the medium on which the organism was grown the inhibiting influence was destroyed. This explanation was rejected by Olitzky³ in the study of *Ps. fluorescens*, and later by Horrocks,⁷ who took gelatin slopes from which the growth of *Ps. fluorescens* had been removed, melted

* P. 34. Unfortunately the copies of the Local Government Board Report containing this have not been available to me, and I have been unable to determine on what experimental evidence Dr. Klein bases this explanation.

them up, and neutralized the gelatin without favoring the subsequent growth of *B. typhosus*.

In this work it has been found that all of the mixtures in which the collodion sac were used were strongly alkaline, varying from -0.7 to -1.2 (Fuller's scale); *i. e.*, they required from 0.7 to 1.2 c.c. $n/20$ HCl to neutralize 5 c.c. of the culture medium. That this alkalinity, as hydroxyl ions, would not in itself account for the death of *B. typhosus* seems certain, since it has been found repeatedly that the typhoid germ can grow luxuriantly where sodium hydrate (NaOH) is present so as to give a reaction of -1.5 . All of the micro-organisms which have been found to antagonize *B. typhosus* are strong alkali producers, but in no case do they produce a greater quantity of alkali than that which corresponds to 15 c.c. of normal sodium hydrate per liter of medium, and, as stated above, this degree of alkalinity permits the ready growth of *B. typhosus*, so that it seems evident that it is not simply the alkalinity of the medium which produces the antagonism in the case studied here.

GENERAL CONCLUSIONS.

The most important of the conclusions which have been reached as the result of this study may be summarized as follows:

1. There is a marked antagonism exerted by mixed cultures of bacteria obtained from the soil and water on *B. typhosus* when the same are grown in broth and a collodion sac containing the typhoid germ is immersed therein.
2. This antagonism results in not merely checking the growth, but in actually killing the typhoid germs. In many cases the killing off amounts to extinction.
3. The death-rate of *B. typhosus* is more or less rapid, depending on the period of preliminary cultivation of the antibiotics.
4. There is no evidence to show that the antagonistic substances exist ready-formed in the soil or water, but rather that the antagonism depends on the rapid development of the germs in the immediate presence of *B. typhosus*.
5. These antagonistic bacteria are widely distributed in nature, being present in various types of soils and waters.

6. An antagonism has been definitely associated with several different species of bacteria, viz., *B. vulgatus*, *B. vulgaris*, *Ps. fluorescens*, and *Ps. putida*. Of these the first-named is assigned this rôle for the first time; the second has been suspected of producing an antagonism; while the third and fourth have been associated with this phenomenon by previous observers.

7. Changes in the environment of these organisms, such as temperature, oxygen supply, reaction of medium, amount of dextrose, etc., seem to have little or no influence on the production of the antagonistic substances. In other words, whenever the environment is such that a good growth of the organisms occurs, the antagonistic substances are apparently always produced.

8. The energy with which the antagonistic substances act depends on the temperature. At 38° C. the action is very pronounced. At the temperature of the ice-chest (10–12°) the typhoid germ may grow in the by-products of the other germs, which at higher temperatures are quickly fatal.

9. The antagonistic substances are thermo-stable, being able to withstand a temperature of 120° C. for at least ten minutes.

10. The cause of the antagonism is not due, in the cases studied, to the exhaustion of the food supply, the action of proteolytic enzymes, specific poisons, or the production of hydroxyl ions simply.

11. This antagonistic action is not due to any peculiarity of a single typhoid culture, but is equally noticeable in at least three different strains.

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EXPLANATION OF PLATE XVIII.

FIG. 1.—Double-plate method of studying antagonism. The Petri dish is divided into two halves by means of a small glass tube which is sealed to the dish with collodion. In one half is poured sterile agar, and into the other half agar thickly seeded with the antibiont. After this has been incubated for some time, in this case forty-eight hours, streaks of the *Bacillus typhosus* are made across the surface of the entire plate. The photograph is taken forty-eight hours later. There is practically no growth of *B. typhosus* on the infected parts of the plates. *A* is *Bacillus vulgaris*; *B* is *Bacillus vulgaris*; *C* is *Pseudomonas fluorescens*; and *D* is *Pseudomonas putida*.

FIG. 2.—Agar-block method of illustrating antagonism. *A*, sterile bouillon showing typhoid streak, one month old; *B*, heated culture of *Pseudomonas fluorescens*, no growth, same age as *A*; *C*, sterile bouillon one week old growth; *D*, *Bacillus vulgaris*; *E*, *Bacillus vulgaris*; *F*, *Pseudomonas fluorescens*; *G* *Pseudomonas putida*, showing slight growth. Cultures a week old when not otherwise stated.

THE LONGEVITY OF THE TYPHOID BACILLUS IN WATER.*

E. O. JORDAN, H. L. RUSSELL, F. R. ZEIT.

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INTRODUCTION.

THE writers of this paper were requested by the Sanitary District of Chicago, in the fall of 1903, to conduct some experiments upon the life of the typhoid bacillus in the waters of Lake Michigan, the Chicago Drainage Canal, and the Illinois River. The purpose of these experiments was to shed light upon the question as to whether the typhoid bacillus could survive the passage from the Chicago Drainage Canal to the mouth of the Illinois River, under the conditions obtaining in the latter stream. The particular circumstances leading to this inquiry have been fully set forth by one of us elsewhere¹ and need not be here rehearsed.

Previous experiments that have been made to determine the longevity of the typhoid bacillus in water have been conducted, for the most part, if not wholly, in glass vessels in a laboratory, and have been carried on under such varied conditions of temperature, character of water, and number and age of bacteria introduced, that widely divergent conclusions have been reached by different observers. From these experiments, however, substantial accord has resulted on two points: first, that typhoid bacilli die out more

* Received for publication May 10, 1904.

¹ JORDAN, *Jour. Exper. Med.*, 1901, 5, p. 271.

rapidly in unsterilized water than in the same water sterilized by heat: second, that when typhoid bacilli are introduced into unsterilized water containing little organic matter, their longevity is more prolonged than in water charged with considerable organic matter.¹ As regards application of results hitherto obtained to particular cases the available evidence appears to indicate that the conditions of laboratory experiments are so far removed from the conditions found in nature as to afford conclusions of doubtful value. Especially in experimental work designed to test the so-called self-purification of streams it would seem desirable to conform to natural conditions, so far as practicable, in order to secure any very cogent evidence.

Division of the work.—The work at the several stations selected for observation was definitely assigned to individuals who were responsible for the carrying out of details. In this way practically three independent experiments were executed simultaneously with the same strains of typhoid cultures. The details of the work were varied somewhat, yet the methods pursued were essentially the same in all cases.

The proximity of four of the experiment stations to Chicago rendered it possible to conduct the work in the laboratories of the Northwestern University and the University of Chicago. Acknowledgment is made in this connection of the services of those who have been engaged in various parts of the investigation.

Experiments upon Lake Michigan and Chicago River water were carried out by Professor F. R. Zeit, with the co-operation of Dr. V. H. Bassett. Dr. Bassett also added some independent experiments on the longevity of the dysentery bacillus, which are incorporated in this paper.

Experiments on the Drainage Canal at its upper (Robey Street) and lower (Lockport) ends were conducted by Professor E. O. Jordan, with the assistance of Dr. Norman MacLeod Harris, Dr. Ernest E. Irons, and Miss Mary C. Lincoln. We are especially indebted to Dr. Harris for the preparation and testing of the special culture media used in the work of isolation. A special report by him on the comparative value of the media will follow in a later number of this JOURNAL.

The studies on the Illinois River, under the direction of Professor H. L. Russell, were carried out in the biological laboratory of the Bradley Polytechnic Institute at Peoria, Ill., the facilities of which were kindly placed at our disposal by Professor Wales M. Packard. The work of securing the samples and the isolation of presumptive typhoid colonies was done by Mr. E. G. Hastings. The cultures were then sent to the University of Wisconsin at Madison, where they were subjected to further study and identification by Professor Russell, who was assisted in this work by Professor W. D. Frost and G. J. Marquette.

¹ E. g., FRANKLAND, *Ztschr. f. Hyg.*, 1895, p. 19, 3, 3.

TECHNIQUE.

Isolation of cultures.—It was considered important to employ freshly isolated typhoid cultures in the experiments, since one of the writers¹ had shown that a difference exists, as regards longevity in water, between freshly isolated strains and those kept under cultivation for some time. Three cultures, designated respectively as *B. typhosus* "x," "y," and "z," were accordingly procured for the purpose.

B. typhosus x was isolated on September 22, 1903, from the blood of a typhoid fever patient. The symptomatology of this case was typical, and the bacillus was obtained in pure culture from the blood. The case terminated fatally, and the autopsy showed the characteristic lesions.

B. typhosus y was isolated October 1, 1903, from the urine of a typhoid fever patient, showing typical symptoms, and responding positively on the tenth day of the disease to the agglutination test. This bacillus was used in pure culture for some inoculations, and the urine of this patient, containing typhoid bacilli, was also used for direct inoculation into some of the sacs.

B. typhosus z was isolated September 28, 1903, from the feces of a typhoid fever patient. The case was perfectly typical and terminated fatally.

These three strains were critically examined as regards all of their biological characters, and proved to be thoroughly typical cultures of the typhoid bacillus. All three agglutinated in high dilutions (1:1,000) with the serum of a rabbit immunized with the University of Chicago laboratory stock culture of *B. typhosus*.²

The use of these newly isolated strains, and their introduction, both directly and in the urine of the typhoid fever patient, into the waters studied, was thought to afford a reasonably close approximation to natural conditions.

Method of exposing bacilli to canal and river waters.—The organisms in a flowing stream are subjected to a more or less changing environment, varying according to local circumstances; hence, if natural conditions were to be precisely imitated, it would be necessary to subject the bacteria to these changing conditions. Manifestly such a course as this would be difficult to carry into effect, but the difficulty may be partly overcome by selecting for experi-

¹ JORDAN, *Med. News*, Sept. 28, 1895.

² These freshly isolated cultures did not, however, agglutinate in as high dilutions as typhoid strains that had been under cultivation in this laboratory for some months or years. These latter agglutinated in dilutions of about 1:10,000 with this same serum.—E. O. J.

mentation different points along a stream, choosing these so as to represent wide ranges in conditions. In the present case a great difference in conditions exists between the highly polluted Chicago River or the upper portion of the Drainage Canal, and the purified water in the lower stretches of the Illinois River. Accordingly the following stations were selected for experiment:

TABLE I.

No.	Station	Distance from Lake Michig'n	Time of Flow
		Miles	Days
1.....	Lake Michigan (tap supply at 2400 Dearborn St. derived from "Four-Mile Crib").....	0	0
2.....	Chicago River, Ashland Ave.....	5½	..
3.....	Head of Drainage Canal, Robey St., Chicago....	6	..
4.....	Mouth of Drainage Canal, Bear Trap Dam, Lockport, Ill.....	34	2
5.....	Illinois River, Averyville.....	125	5-8

By exposing the several strains of typhoid organisms to these various waters it was thought that the range in conditions would be nearly as great as would be encountered in the natural passage downstream.

Use of permeable sacs.—The errors introduced by using ordinary glass containers in work of this character have been clearly shown by Ficker,¹ and again very recently by Hesse.² In our experiments the attempt has been made to expose the typhoid organism under as nearly natural conditions as possible, taking into consideration the difficulties which necessarily obtain in recovering the organism from the water. For this purpose permeable sacs of parchment or celloidin were employed, which were filled with canal or river water taken at the respective stations, and then infected with typhoid bacilli.

Celloidin sacs.—Celloidin capsules were first used in bacteriological work by Morpurgo and Tirelli in 1892³ for the cultivation of tubercle bacilli. The method has since been used and perfected by various investigators with more or less success. Metchnikoff, Roux, and Salimbeni⁴ used it for their studies of cholera toxin; Nocard and Roux,⁵ for their studies of pleuropneumonia in cattle; and Nocard,⁶ in his attempt to transform human into avian tubercle bacilli. McCrae⁷ in his studies of agglutination devised a simpler

¹ *Ztschr. f. Hyg.*, 1895, 29, p. 1.² *Ibid.*, 1904, 46, p. 1.³ *Archives Ital. de Biol.*, 1892, 18, p. 187.⁴ *Ann. de l'Inst. Past.*, 1896, 10, p. 257.⁵ *Ibid.*, 1898, 12, p. 270.⁶ *Ibid.*, 1898, 12, p. 561.⁷ *Jour. Exper. Med.*, 1901, 5, p. 635.

and more practical method, which was still further perfected by Harris.¹

The successful use of celloidin sacs in bacteriology by these investigators led one of us (F. R. Z.) to employ Harris's method in these experiments.

The sac became somewhat brittle and less flexible after about ten days' exposure in the water. Breaks occurred but rarely, and then only at the joint of the glass tube and sac, or along the middle of the sac, where the original joint of the gelatin capsule had been. In the river the sacs became coated with a slimy deposit in about five days, but the integrity of the sac was not affected by it for several weeks.

These celloidin sacs and their contents were suspended in running tap water (Lake Michigan water) in a large glass receptacle containing six liters of water, one liter of which was replaced every one or two minutes. The glass tubes were cotton-stoppered, and held by burette clamps so that the sacs and the lower portion of the glass tubes were submerged.

The celloidin sacs used in the Chicago River were sealed and immersed in the water. They were protected from floating débris by being inclosed in a box, to which the water had free access.

The sacs were tested by submerging them in water, blowing into the glass tubes, and looking for air bubbles. If no air bubbles were detected, they were filled with a watery solution of egg albumin and suspended in a small jar containing water for twenty-four to forty-eight hours. The outside water was tested for albumin. After the integrity of the sacs was established by these two tests, their osmotic property was tested by solutions of salt and sulphate of magnesia.

For the collection of samples of river water it was found necessary to bring one entire sac to the laboratory daily. In the case of the Lake Michigan tap water, we were able, due to the conveniences of conducting the experiments in the laboratory, to take out small portions by means of a sterile capillary pipette.

Although the method of using permeable sacs made of celloidin has been employed for several years, so far as we are aware this technical method has not before been applied to a study of the present character. In addition to using celloidin sacs, another method was practiced in which the typhoid cultures were exposed in sacs made of vegetable parchment, such as are commonly employed in laboratories for dialyzing work. This method has been previously used by one of us,² with success, in studying the influence of certain environmental factors on growth of milk bacteria.

Dialysis occurs more or less rapidly in such sacs, and therefore any disturbance in equilibrium is quickly restored by osmosis. Dialyzable substances in the water, either inside or outside of the

¹ *Bull. Johns Hopkins Hosp.*, 1902, 13, p. 112.

² RUSSELL, *Eighteenth Report, Wis. Agr'l. Exper. Sta.*, 1901, p. 165.

sac, would therefore be able to pass in or out. If the water outside of the sac was rich in organic matter and bacteria, and consequently contained a large amount of soluble products formed by these organisms, the osmotic pressure might result in an inflow that would continue until equilibrium was established. On the other hand, if abundant growth occurred in the sac, such as is usually observed when a small quantity of water is confined in a glass bottle, the effect of this excessive growth might be diminished by the outflow of such soluble growth products. By establishing such a state of equilibrium it would seem that the conditions within the sac must approach much more nearly to those which obtain in the flowing stream than would be possible where diffusion or osmosis is excluded.

Parchment sacs.—The parchment used in this work is purchased in the form of long tubes with a diameter of about 2.5 inches. This tubing was cut into lengths so that each sac would hold from 200 to 700 c.c. of liquid. In the experiments in the Illinois River at Averyville (H. L. R.) the sacs were made by folding about 6 inches of the tubing back and forth on itself, making at least eight thicknesses, which was then clamped tightly with strong clamps. In the Averyville experiments both ends of the sacs were closed.

In the experiments in the Drainage Canal (E. O. J.) the tubes were closed at one end by folding a number of times and then sealing tightly with a mixture of resin and tallow (8:1). The upper end of the sac was fastened to a piece of glass tubing $\frac{5}{8}$ inches in diameter and 6 inches long, the lower end of which was drawn out so that a firm union was made with the parchment by binding the moistened parchment around the tube at the point of constriction. The lower end of the parchment section of the tube (22 inches long) was sealed by turning up the end ($1\frac{1}{2}$ inches), rolling it tightly and wrapping with thread. Both the upper and lower joints were saturated with a mixture of resin and tallow. The capacity of the completed tube was 700 c.c. After about three or four days in the water, the sacs became coated with a dark, slimy, later shaggy, material, which on microscopic examination was seen to consist of a species of bryozoa. The same growth is found on all submerged planks and debris in the vicinity. This growth was most marked at Robey Street (upper end of Drainage Canal), but was seen to a less degree at Lockport (lower end).

The sacs exposed in the Illinois River at Averyville began to show small circular spots on the outside after they had been in place four or five days. These areas gradually increased in size for a time, but never became confluent. An examination showed the hyphal filaments of a pinkish fungus. In the course of about two weeks the portions of the parchment sac affected in this way softened under the development of cytolytic enzymes, so that the sacs were quite readily ruptured. After losing several sacs in this way in the

course of eleven or twelve days, the contents of the remaining sacs were transferred to new tubes after they had been immersed about a week.

When properly prepared, this parchment tubing is firm and tough, and capable of withstanding considerable rough treatment. Care must be taken, however, in selecting the sacs, as they are frequently defective along the seams. The sacs were first tested by filling and suspending in air, rejecting any which showed any signs of leakage. Their integrity was also tested in the following way: Sacs were filled with water, then immersed in cylinders filled with water, after which the whole apparatus was sterilized in the autoclave. In some cases the outside water was inoculated with typhoid bacilli, and in others the infection was made on the inside of the parchment sacs.

In carrying out the experiments in the water of the Drainage Canal, the parchment-sacs were suspended within a frame covered with wire screen. This frame was made of pine strips and was four feet long, three feet wide, and three and one-half feet deep. The bottom, top, and lower five-sixths of the sides and ends were covered with wire screen; the upper six inches of the sides and ends of the box, and the sides and ends of the cover, which were six inches deep, were made of one-inch pine fencing. The top opened on hinges, and was fastened in place by hasp and padlock. The box, as a whole, was so placed that it floated with the water-level at about the lower margin of the upper solid half of the body. The part above water was thus of boards and served to protect the contents from waves made by passing vessels or by high winds.

Within the box were several cross strips about one inch above the water-level, in which, at intervals of about two inches, slots were cut to receive the glass ends of the tubes. Opposite each slot was a staple through which was passed a cord attached to the sac to hold it securely in place. To guard further against the tipping of the sacs, small strips were fastened across the slots so as effectually to prevent accidental dislodgment. At the lower end of each sac a small lead weight was attached which kept the sac in a vertical position in the water. A cord was also fastened to the lower end by which the sac was brought to the surface and its contents agitated at the time of taking samples. The sacs were open to the air at the upper end, through the glass tube, allowing free interchange of air and gases. Owing to the slight wave-motion usually present, there was a fairly constant, though slight, movement of the body of the sacs.

Two points in the Drainage Canal were selected for carrying out the experiment, one box being placed in the water at Robey Street near the mouth of the canal, and the other at Lockport near the Bear Trap Dam, twenty-eight miles from Robey Street. In all the experiments the sacs were filled with water taken from the Drainage Canal at the point where the experiment was being performed; and into the sacs thus filled typhoid bacilli were immediately introduced. Several sacs were always inoculated at the same time and in the same way, to avoid the danger of a break in the experiment through possible mischance.

Owing to the frequency of high waves in the Illinois River at Averyville, it was found necessary to submerge the sacs about six inches below the surface of the water. The sacs were placed in galvanized iron cylinders, the top

and bottom of which were covered with a coarse wire netting, to which the sacs were firmly clamped. The covers to these cylinders were padlocked, and the whole apparatus was lowered into the river at midstream, just above the Averyville wagon bridge.

Seeding the sacs with typhoid cultures.—The sacs were first filled with the respective waters taken from the different stations, and then seeded with homogeneous suspensions of fresh cultures of the different typhoid strains. The range used in the Lake Michigan and Chicago River experiments was from 500 to 2,000,000 typhoid bacilli per c.c.; in the Drainage Canal, 180 to 857,000 per c.c.; and in the waters of the Illinois River, from 540 to 20,400 per c.c. The data as to the seeding are supplied in more detail in connection with the tabular matter later presented.

It is important to determine the absolute number of typhoid organisms exposed to the influence of the river water, but it is perhaps more important to note the relation that exists between the bacteria normally present in the various waters and the typhoid bacilli introduced. The extent of typhoid seeding should therefore be studied from the relative as well as the absolute point of view.

The sewage-polluted waters of the Chicago River and the Drainage Canal contain a varying, but always a large, number of organisms, while the waters from the Lake and the Illinois River are relatively low in bacteria. From observations made on the germ-content of the river and canal, the following data as to the average bacterial content of the water at the different stations are presented:

TABLE II.

NORMAL BACTERIAL CONTENT OF WATERS TAKEN AT DIFFERENT STATIONS,
COMPARING WITH TYPHOID SEEDINGS.

STATIONS	BACTERIA PER CUBIC CENTIMETER	
	Water and Sewage Forms	Typhoid Bacilli Added.
Lake Michigan.....	68- 2,000	540-1,184,000
Chicago River.....	80,000-1,500,000	500-2,000,000
Drainage Canal (Robey Street).....	100,000- 400,000	670- 857,000
Drainage Canal (Lockport).....	100,000- 400,000	180- 360,000
Illinois River (Averyville).....	1,800- 4,000	540- 20,400

It is evident from the foregoing that the number of typhoid bacilli introduced into the respective waters was usually a large proportion of, and often surpassed, the number of bacteria of all kinds naturally found in such waters.

Method of isolating the typhoid bacilli.—Particular attention was given to the problem of isolating typhoid bacilli in the presence of sewage bacteria. Many media have been proposed for this purpose, and a number of the most promising were tested with a view to possible utilization.

One of us (F. R. Z.) made a comparative study of the value of the following methods for the detection of small numbers of typhoid bacilli in badly polluted waters, which will be the subject of a future paper:

1. Special culture methods.
 - a) Drigalski-Conradi method.¹
 - b) Drigalski-Conradi agar plates, followed by subcultures in Hiss tubes.
2. Direct agglutination method for large quantities of water.
 - a) Hagemann method.²
3. Enriching methods with agglutination.
 - a) Schepilewsky's modification of Windelbandt's method.³
 - b) Altschüler's method.⁴
4. Chemical precipitation method for large quantities of water.
 - a) Schüder.⁵

Of these the Drigalski-Conradi method, followed by subcultures in Hiss tubes, was used extensively, and gave excellent results where small quantities of water had to be used for examination. Altschüler's and Schüder's methods both proved highly efficient in the examination of large quantities of water for typhoid bacilli.

Thanks are due to Messrs. Parke, Davis & Co., of Detroit, for kindly furnishing us an antiserum of very high agglutinating power, together with cultures of their strain "O" B. typhosus, used in immunizing the horse from which this serum was obtained.

In the work on the Drainage Canal (E. O. J.) four media were chiefly used, viz.:

a) The medium of Drigalski and Conradi.¹ This was modified from the original description of these writers by leaving out the nutrose, sodium chloride, and the solution of water-free sodium carbonate, and by filtering the mixture before adding the litmus lactose solution. In this way a much clearer medium was secured which contained scarcely any flocculent precipitate after being sterilized thrice in the steam bath.

b) A modification of MacConkey's medium.⁶ At first the original MacConkey medium, as modified by Grünbaum and Hume, was used, but this was found to have very little restraining power upon the sewage bacteria, so

¹ *Ztschr. f. Hyg.*, 1902, 39, p. 283.

² *Centralbl. f. Bakt.*, 1903, 33, Apr. 22, p. 743.

³ *Ibid.*, Feb. 6, 1903, 33, p. 394.

⁴ *Ibid.*, Apr. 22, 1903, 33, p. 741.

⁵ *Ztschr. f. Hyg.*, 1903, 42, p. 317.

⁶ *Brit. Med. Jour.*, 1902, Pt. I, p. 1473.

that crystal violet solution (1 g. to 100 c.c.) was added in the proportion of 83 c.c. per liter of the medium, and the technique was further altered in the following manner: After the agar and peptone were dissolved, the medium was made 1.8 acid to phenolphthalein by adding a sodium hydrate solution, and the mass was autoclaved for five minutes at 120° C., cooled, cleared with white of egg, and filtered through cotton. This treatment prevented the formation of the flocculent precipitate which caused trouble in the original method. Finally the other ingredients were added and also the crystal violet, and the medium was tubed and sterilized once in the Arnold sterilizer for thirty minutes.

c) Horrock's glucose litmus agar.¹ Two per cent. glucose peptone agar was made in the usual manner, and sufficient aqueous solution of litmus was added to give the medium a light blue color. For use the medium is melted, cooled to 42° C. and then decinormal sodium hydrate added so that each 10 c.c. of the medium has an alkalinity of 1.8 c.c. decinormal alkali. This medium differentiates well, but the abundant gas formation impairs its usefulness for the conditions under which we worked.

d) The plating medium of Hiss. This was prepared according to the description given by the author.² Although not restraining the development of sewage forms in the way that the other media did, it proved one of the most useful methods, particularly when the plates were not too thickly crowded with sewage bacteria. Differentiation of the surface colonies was usually very sharp.

Other media, notably Elsner's potassium iodide medium³ were tested, but proved very inferior to the four media above described; these four alone were used in the routine work.

In the work on the Illinois River at Averyville (H. L. R.) the Drigalski and Conradi medium was exclusively employed, differentiation being here less of a problem than in the waters charged with sewage bacteria.

EXPERIMENTS CONDUCTED IN LAKE MICHIGAN WATER AND IN THE CHICAGO RIVER (F. R. Z.).

A series of experiments upon the longevity of the typhoid bacillus in Lake Michigan water, made during the fall of 1897 and the spring of 1898, had convinced the writer that typhoid bacilli die much more rapidly when free in nature than had been generally believed. Although the conclusions drawn were open to the criticism that in the liter flasks, used in experiments to hold the artificially infected lake water, the conditions were different from those existing in nature, the results compare very well with those obtained in our experiments with parchment and celloidin

¹ W. H. HORROCKS, *An Introduction to the Bacteriological Examination of Water* (London), 1901, p. 205.

² *Jour. Med. Resch.*, 1902, 8, p. 148.

³ *Ztschr. f. Hyg.*, 1895, 21, p. 25.

sacs suspended in Lake Michigan tap water, under the most favorable conditions for the diffusion of saprophytic products.

The experiments consisted in seeding liter flasks containing Lake Michigan tap water with varying numbers of typhoid bacilli, and studying the effects of temperature, light, and the addition of saprophytic products (sewage) upon the longevity of typhoid bacilli.

Similar experiments were undertaken with the water of the Mississippi, Missouri, and the Illinois Rivers at Grafton, between the months of October, 1901, and March, 1902, and it was found that the more polluted a water was and the more saprophytes were present, the sooner would the typhoid bacilli disappear.

The natural objection to these flask experiments, that the accumulating products of this saprophytic overgrowth were the cause of the rapid disappearance of typhoid bacilli, and that the results could not, therefore, be applied to conditions naturally existing in lake or river water, has not been justified by the results of the experiments to be related, because typhoid bacilli have died in our parchment and celloidin sacs, under the most favorable conditions of diffusion, in the natural flowing water, in as short, or even a shorter, time than they did in the same waters when contained in glass flasks at temperatures ranging from 12° to 20° C.

The present experiments give first the results with glass bottles as containers, in which diffusion of bacterial excretion products is excluded; and next, the results when diffusion of excretion products is favored by the use of parchment and celloidin sacs.

I. EXPERIMENTS WITH LAKE MICHIGAN TAP WATER.

In the first five of the following fifteen experiments with Lake Michigan tap water, glass jars or bottles were used as containers; in the next seven, parchment sacs; and in the last two, celloidin sacs, the parchment and celloidin sacs being suspended in running tap water.

Experiment 1.—Eight hundred c.c. of raw (unheated) tap water, taken from a tap in Dr. Zeit's laboratory at the Postgraduate Hospital, were placed in a glass jar, and one loopful of a twenty-four-hour agar culture of *B. typhosus* (Race 0 from Parke, Davis & Co.) was added, the seeding being

estimated by plating to be approximately 1,000,000 per cubic centimeter. The bacterial count of the water at the time of seeding was 85 per cubic centimeter. The temperature of the water varied from 7° to 16° C. Above, the jar was open to the air, which entered freely through the cotton stopper.

Samples were taken daily, after shaking the jar to insure thorough mixing, a portion of the contents being taken with a sterile pipette. Plate cultures were made in plain neutral agar, with a seeding of from 1.1.000 to 5 c.c., according to the bacterial content. These cultures were incubated at 39° C. After twenty-four to forty-eight hours the plates were carefully examined and the typhoid-like colonies cultured in Hiss's tube medium. Cultures in this medium which resembled those of the typhoid bacillus were further examined, and their identity established by the specific agglutination test of Widal, and also by further culture methods. From the tenth to the fourteenth day all the colonies appearing on the plates were examined, with negative results, and the complete disappearance of the typhoid bacillus was demonstrated. For comparison, plate cultures of the daily samples were made and incubated at 25° C., and the bacterial content per cubic centimeter (after three days), and the character of the growth, compared with that of the plate cultures at 39° C.

The results of the experiment are shown in Table III.

TABLE III.

Day of Experiment	Temperature	Bacteria per c.c. of Tap Water	Bacterial Count of Plates Incubated at 39° per c.c.	Bacterial Count of Plates Incubated at 25° per c.c.	B. typhosus + = present 0 = absent
Seeding	9° C.	85	1,737,000	+
1	14	90	735,000	1,027,000	+
2	13	109	711,000	1,175,000	+
3	16	100	339,000	360,000	+
4	16	180	71,000	154,000	+
5	9°	38	5,800	28,600	+
6	10	80	9,300	12,400	+
7	9	48	1,600	3,200	0
8	12	48	5,000	4,700	0
9	12	76	360	1,000	0
10	9	80	78	8,000	0
11	8	148	197	760	0
12	7	128	41	1,260	0
13	16	120	72	1,820	0
14	8	110	0	2,180	0

NOTES.—On the eighteenth and twentieth days no colonies developed in plates seeded with 1 c.c. of the water, and in plates seeded with 5 c.c. only a few colonies developed at 39° C. On the seventh, eighth, and ninth days no typhoid-like colonies developed on the plates. From the tenth to the fourteenth days, inclusive, all the colonies on the plate cultures were examined.

Experiment 2.—In this experiment four loopfuls of the water of condensation were used, and the flask was placed in an earthen jar and surrounded by flowing water kept at a uniform temperature (19° C.) for twelve days. The germ content was 180 per c.c. and plating after seeding with *B. typhosus*, 2,200,000 per c.c.

Plates poured with Drigalski-Conradi¹ agar (about 3 mm. thick) remained open for an hour until the agar became hard. After shaking the flask containing the water to be examined, a glass rod, bent at an angle, sterilized, was dipped into the water and smeared over the first plate. Dilutions were made by smearing a second, third, and fourth plate. These plates remained open until perfectly dry (one-half to one hour), and were then incubated at 37° for twenty-four hours. All discrete blue colonies were subcultured in Hiss tubes and grown at 20°. All non-gas-forming, uniformly clouded Hiss tubes were tested by Parke, Davis & Co.'s immune serum for agglutination.

Typhoid bacilli were found by this method on the third, fifth, sixth, and seventh days, but none on the eighth or ninth days.

On the twelfth day Altschüler's² method was used. Ten grams of peptone and 5 g. of sodium chloride were added to the contents of the flask which was then incubated for twenty-four hours at 37° C. Fifty c.c. of the upper stratum of the flask contents were placed in a sterile burette, and 1 c.c. of Parke, Davis & Co.'s serum was added. This was set aside for seven hours. No precipitate was visible. One c.c. was drawn off into another burette containing 50 c.c. of sterile peptone (0.5 g.) and sodium chloride (0.25 g.) solution and a few millet-seed-sized pebbles. This burette was shaken to break up clumps and incubated at 37° for twenty-four hours. Drigalski-Conradi plates were smeared and incubated as above; blue colonies were subcultured in Hiss tubes. No non-gas-forming, uniformly clouded Hiss tubes.

Experiment 3.—Fresh feces of a healthy person were stirred into 1,000 c.c. of raw tap water until the turbidity was such that print became indistinct when viewed through the solution in a 1,000 c.c. cylindrical graduate. This mixture was subjected to the same conditions of seeding and temperature as in the last experiment. The immediate plating in gelatin was 5,800,000 per cubic centimeter. Examination, as in the last experiment, showed typhoid bacilli on immediate plating, but none on the first, third, or eighth days thereafter.

As a control for this experiment two 1,000 c.c. flasks were prepared with human excreta as above. One (A) was seeded with four loopfuls of the usual culture of *B. typhosus*; the other (B) received no typhoid bacilli until it had been kept twenty-four hours at a temperature of 15° C., when it was seeded with 1:1,000 loop of the same culture as Flask A, after which both flasks were examined by Schüder's method.³

To each flask were added 10 c.c. of a 7.75 per cent. solution of hyposulphite of soda and 10 c.c. of a 10 per cent. solution of nitrate of lead. After standing for twenty-four hours the clear fluid was decanted, 7 c.c. of a 100 per cent. solution of hyposulphite of soda added to the sediment, and the flask shaken. After standing a short time, the insoluble particles settled. Drigalski-Conradi smears were made and examined as in the preceding experiments, from the upper clear strata.

In Flask A, seeded with four loopfuls of typhoid culture twenty-four hours before precipitation, typhoid bacilli were absent, while in Flask B, seeded with only 1:1,000 loop just before precipitation, the *B. typhosus* was found.

¹ *Ztschr. f. Hyg.*, 1902, 39, p. 283.

² *Centrbl. f. Bakt.*, 1903, 33, p. 741.

³ *Ztschr. f. Hyg.*, 1903, 42, p. 317

Schüder's method is of much value, as it enables us to precipitate all the bacteria in a larger quantity of water, and it allows the detection of typhoid bacilli in water highly polluted with saprophytes. Although the growth of saprophytes is much inhibited, typhoid colonies were numerous in plates from Flask B, despite the fact that the seeding of typhoid bacilli was only 1/1,000 loop with nearly 6,000,000 per c.c.

Experiment 4.—In this experiment sterilization for thirty minutes at 120° in the autoclave preceded the seeding, and the flask was kept in flowing tap-water in a uniform temperature (19° C.) for forty days. The results are shown in the following table:

TABLE IV.

Direct plating - - - - -	1,500,000 typhoid bacilli per 1 c.c.
After 5 days - - - - -	350,000 " " " "
After 10 days - - - - -	110,000 " " " "
After 15 days - - - - -	10,000 " " " "
After 20 days - - - - -	30 " " " "
After 25 days - - - - -	12 " " " "
After 30 days - - - - -	0 " " " "
After 35 days - - - - -	0 " " " "
After 40 days - - - - -	0 " " " "

Experiment 5.—In this experiment a four ounce bottle stopped with a sterile cotton plug was used instead of the liter flask, and the tap water was rendered germ-free by filtration through sterilized porcelain (unglazed). The germ content previous to filtration was 100 per cubic centimeter. Cultures of 5 c.c. of the filtered tap water in bullion remained sterile. To 100 c.c. of this sterilized tap water was added 1 c.c. of a 1:10,000 dilution in sterile salt solution of a twenty-four hour agar culture of *B. typhosus* Race No. 500.2 (a culture obtained from the Johns Hopkins Pathological Laboratory). This seeding was estimated by plate methods to be about 500 typhoid bacilli to 1 c.c. The bottle containing the infected water was placed in a thermostat maintained at a temperature of about 20° C. Free entry of air was allowed through the cotton plug; light was excluded.

The bottle was removed daily, and, after shaking gently to insure even seeding, plate cultures in agar-agar were seeded with 1 c.c. of the infected water. These cultures were incubated from twenty-four to forty-eight hours, and the typhoid-like colonies were then subcultured in Hiss tubes and identi-

TABLE V.

Day of Experiment	Amount of Water Plated	No. of Typhoid Bacilli Found	Estimated No. in Bottle	<i>B. typhosus</i> + = present 0 = absent
Seeding	1 c.c.	500	50,000	+
1	1	4	396	+
2	1	1	98	+
3	15	2	11	+
4	30	1	3	+
5	15	0	0	0
6	10	0	0	0
7	5	0	0	0
8	5	0	0	0
9	5	0	0	0
10	12	0	0	0

fied by means of further culture, and the Widal reaction. As the typhoid organisms disappeared, larger amounts of water were used for seeding. During the last three days of the experiment all the water remaining in the bottle was plated out, but no typhoid organisms were found. The results are presented in Table V.

Instead of the glass containers, parchment sacs were used in the next seven experiments.

Experiments 6 and 7.—In these experiments, where the bacterial count before the seeding was estimated to be 2,000 per cubic centimeter, and from a few hundred to a few thousand, respectively, the 800 c.c. of tap water was seeded with a small loopful of a twenty-four-hour culture of *B. typhosus*, Race "y," received from Dr. E. O. Jordan, giving a seeding of about 540,000 in the one case and of 450,000 in the other. The parchment sacs were subjected, in both experiments, to the same conditions as those surrounding the glass container in Experiment 1, already described. Samples of the infected water were taken daily and subjected to the usual cultural and agglutination tests. Typhoid organisms were found daily up to the seventh day in Experiment 6, and up to the fifth day in Experiment 7, but none thereafter. In both cases there was a marked change in the character of the plates in the latter part of the first week. In Experiment 7 a single culture on the fifteenth day showed the presence of only a few saprophytic organisms.

Experiment 8.—This was in all respects as Experiment 1, except that a parchment sac was used in place of the glass container. At the end of this experiment the parchment sac was examined and found to be intact. It appeared to be as strong as when first put into the water, and there was little or no deposit on its surface. The results of this experiment are shown in Table VI.

TABLE VI.

Duration of Experiment in Days	Bacterial Count per c.c. at 25° C.	Bacterial Count per c.c. at 39° C.	Ratio Growth at 25-39° C.	<i>B. typhosus</i> += present 0 = absent
Seeding	1,112,000	+
1	1,035,000	+
2	964,000	372,000	2.59	+
3	855,000	576,000	1.46	+
4	616,000	553,000	1.11	+
5	536,000	316,000	1.69	+
6	237,000	205,000	1.16	+
7	103,000	201,000	0.51	+
8	134,000	197,000	0.68	0
9	72,000	23,000	3.10	0
10	32,000	4,400	7.27	0
11	13,000	3,400	3.82	0
12	7,100	74*	9.59	0
13	6,000	34*	17.64	0
14	10,600	100*	10.60	0

NOTES.—On the twelfth, thirteenth, and fourteenth days all the colonies on the plates were examined; *Bacillus typhosus* was not found.

From the first to the fifth day, inclusive, the plates resembled pure cultures of *Bacillus typhosus*, which was recovered with ease.

On the sixth, seventh and eighth days the plates showed mixed cultures of *Bacillus typhosus*, which was secured with increasing difficulty.

From the ninth to the fourteenth day, inclusive, the cultures were saprophytic in character, and the typhoid bacillus could not be found, despite repeated and exhaustive efforts to secure it.

Experiment 9.—This experiment was undertaken as a control to the preceding, the only difference being that the parchment sac in the earlier experiment was surrounded by flowing water, while in Experiment 9 it was suspended in an empty glass cylinder so that the surface of the parchment was freely exposed to the air on all sides. In order to secure temperature conditions similar to those of Experiment 8 the glass cylinder was placed in a large receptacle and surrounded with running tap water. There was free access of air also through the cotton stopper, and the experiment was conducted in the diffuse light of the laboratory. The results are shown in Table VII.

TABLE VII.

Duration of Experiment in Days	Bacterial Count of Plates Incubated at 39° C.	Bacterial Count of Plates Incubated at 25° C.	Bacillus typhosus + = present 0 = absent
Seeding	635,000	+
1	748,000	+
2	711,000	628,000	+
3	340,000	1,836,000	+
4	419,000	2,117,000	+
5	158,000	1,117,000	+
6	142,000	253,000	0
7	13,800	83,000	0
8	316 *	43,000	0
9	1,100 *	11,600	0
10	550 *	2,600	0
11	840 *	6,400	0
12	No growth	11,000	0
13	15 **	5,500	0
14	13 **	8,600	0

NOTES.—On the third, fourth, and fifth days the plate cultures showed a large number of fluorescent organisms. On the sixth to the ninth days inclusive, the fluorescent organisms were numerous, but decreasing in number.

* On the ninth, tenth, and eleventh days all the typhoid-like colonies on the plates were examined.

** On the thirteenth, and fourteenth days all the colonies on the plates were subcultured and examined, but the typhoid bacillus could not be found.

RESULTS.—An overgrowth of saprophytes occurred early in the course of the experiment, and the vital concurrence of these organisms, as well as the action of their products, must be regarded as factors in the death of the typhoid bacillus.

Experiment 10.—In this experiment 800 c.c. of raw tap water, which gave 180 colonies per centimeter in gelatin plates, were placed in a cotton-stoppered parchment sac and seeded with four loopfuls of the water of condensation of a twenty-four-hour culture of *B. typhosus*, Race "O," Parke, Davis & Co. The sac was suspended in running tap water in a large earthenware jar, the temperature of the rapidly flowing water being 1°5-2°5 C. The contents of the sac were examined by Drigalski-Conradi plates and Hiss tubes, with the result of finding typhoid bacilli on the second, fourth, fifth, and sixth days, and with negative results on the seventh and eighth. The Schüder precipitation method was employed as described in Experiment 3, with the balance of the sac contents, on the ninth day, with negative results.

Experiment 11.—A parchment sac was prepared, seeded, and examined as in the preceding experiment. Plating after seeding in gelatin showed 2,300,000 colonies per 1 c.c. Typhoid bacilli were found on the second, fifth, sixth,

and seventh days, but none on the eighth or ninth. On the tenth day Alt-schüler's enriching method was employed, as in Experiment No. 2, with negative results.

Experiment 12.—Eight hundred c.c. of tap water were sterilized by heating for twenty minutes in the autoclave at a temperature of 110° C. The sac was then placed beside the one used in Experiment 8 and subjected to the same conditions. All the other conditions of the experiment, and the method of examining the sac contents for typhoid bacilli, were as in Experiment 8. The result was a steady decrease in the number of typhoid organisms found, but they were still present on the fifteenth day, when the experiment was discontinued. The results are tabulated in Table VIII.

TABLE VIII.

Duration of Experiment in Days	Bacterial Count of Plates Incubated at 39° C. per c.c.	Bacterial Count of Plates Incubated at 25° C. per c.c.	Bacillus typhosus + = present 0 = absent
Seeding	1,184,000	+
1	450,000	618,000	+
2	416,000	716,000	+
3	363,000	510,000	+
4	350,000	904,000	+
5	332,000	814,000	+
6	253,000	366,000	+
7	292,000	316,000	+
8	356,000	474,000	+
9	94,800	94,800	+
10	12,000	83,200	+
11	16,000	150,000	+
21	3,120	28,700	+
13	840	64,000	+
14	+
15	1,000	25,000	+

Experiment 13.—In this experiment a four-ounce glass-stoppered bottle was used. A sterilized salt solution was made by adding 0.6 per cent. of common salt to the tap water and heating at 20° C. in the autoclave. To 100 c.c. this, 1/10,000 of a large loopful of a twenty-four-hour culture of *B. typhosus* (Race No. 500.2, obtained from the Johns Hopkins Pathological Laboratory) was added, the seeding being estimated by plate cultures to be 900 per cubic centimeter. The bottle was placed in the ice-chamber of the refrigerator, the

TABLE IX.

Day of Experiment	Amount of Water Plated	No. of Typhoid Bacilli per c.c.	No. in Bottle Estimated	Bacillus typhosus + = present 0 = absent
Seeding	1 c.c.	900	90,000	+
1	1 c.c.	804	79,500	+
2	1 c.c.	426	41,700	+
3	1 c.c.	326	31,600	+
4	1 c.c.	266	25,500	+
5	1 c.c.	158	15,000	+
6	1 c.c.	145	14,000	+
7	1 c.c.	140	13,000	+
8	1 c.c.	100	9,200	+
9	1 c.c.	80	7,300	+
10	1 c.c.	75	6,800	+

temperature varying from 1° to 8° C. A glass stopper was used, but about 10 c.c. of air were inclosed and renewed daily as the bottle was opened. Light was excluded. Plate cultures were made daily, and as the typhoid bacilli became fewer in number, all the colonies on the plate were examined. The typhoid bacillus was still found on the tenth day, when the experiment was discontinued. The results are presented in detail in Table IX.

Experiment 14.—In this experiment celloidin sacs having a capacity of 30 c.c. each were used in place of parchment sacs. In this case the bacterial count of the tap water at the time of seeding was 68 per cubic centimeter, and the seeding was estimated to be 1,000,000 per cubic centimeter. The infected water was distributed by means of a sterile pipette to the celloidin sacs in amounts of about 20 c.c. The experiment was in all other respects similar to No. 8. The results are presented in Table X.

TABLE X.

Duration of Experiment in Days	Bacterial Count of Plates Incubated at 25° C. per c.c.	Bacterial Count of Plates Incubated at 39° C. per c.c.	Bacillus typhosus + = present 0 = absent
Seeding	1,155,000	+
1	885,000	814,000	+
2	790,000	946,000	+
3	1,146,000	717,000	+
4	757,000	565,000	+
5	320,000	53,600	+
6	292,000	7,000	+
7	514,000	4,000	+
8	558,000	35,600	0
9	600,000	3,800***	0
10	569,000	2,600*	0
11	711,000	3,960	0
12	87,000	182**	0
13	4,030	28**	0
14	3,900	23**	0

NOTES.—On the fourth day numerous fluorescent forms appeared in the plates, especially those incubated at low temperature.

* All the colonies appearing on one-fourth the plate examined with negative results.

** All the colonies appearing on the plates examined with negative results.

*** Negative results obtained by special method. All of the water was removed from one of the capsules, antityphoid serum added in the proportion of 1:100, and the whole placed in a sterile burette for sedimentation. At the end of twenty-four hours the lower portion, about 1 c.c., was removed and plated in neutral agar. The colonies which developed were examined, but the typhoid bacillus was not found.

Experiment 15 differed from the preceding only in that the tap water was sterilized before seeding (as in Experiment 12), and the results confirmed those obtained in Experiment 12, in which a parchment sac was used to contain the sterilized water.

The following eleven experiments, similar in general to those already described with Lake Michigan water, were conducted with the water of the Chicago River:

Experiment 16.—This experiment corresponded in all respects to Experiment 5 above, except that the water of the Chicago River was used instead

of that of Lake Michigan, the germ content before filtration being estimated to be 1,500,000 per cubic centimeter. The results are presented in detail in Table XI.

TABLE XI.

Duration of Experiment in Days	No. of Typhoid Colonies in 1 c.c.	Estimated No. of Typhoid Bacilli in Bottle	Bacillus typhosus + = present 0 = absent
Seeding	496	49,600	+
1	1	99	+
2	0	0	0
3	0	0*	0
4	0	0	0
5	0	0	0
6	0	0	0
7	0	0	0
8	0	0	0
9	0	0	0
10	0	0	0

*On the third day a number of saprophytic organisms appeared in the plate cultures. A control experiment consisting of a culture of 5 c.c. of the filtered water used in beginning the experiment showed that the filtration had not secured an absolutely germ-free water. Diligent search failed to show the presence of the typhoid organism after the first day.

This experiment indicates that if but a small number of the typhoid organisms are present in the river water, their death is a matter of hours rather than of days, and may be independent of the presence of saprophytes in large numbers.

To determine whether the amount of water used in seeding the plates had any deterring effect upon the growth of organisms in the plate cultures, cultures were made from two preparations: (1) 100 c.c. of raw filtered water of the Chicago River, to which was added 1/10,000 part of a loopful of a twenty-four-hour agar culture of *B. typhosus*; (2) 100 c.c. of sterile salt solution seeded with typhoid as in (1). Plate cultures were made of 1 c.c. of each mixture, and after forty-eight hours' incubation at 39° C. the number of colonies was found to be as follows: (1) 496 per cubic centimeter; (2) 500 per cubic centimeter. Repetition of the experiment gave a similar result, so that it was evident that small amounts of the water, when added to nutrient media, had no marked deterrent effect upon the growth of the typhoid bacillus in plate cultures.

Experiment 17.—In this experiment the bottle containing the filtered Chicago River water was placed in the ice-chamber of the refrigerator, the temperature being 1° to 8° C. Glass stoppers were used, but about 10 c.c. of

air was inclosed, and renewed in part daily as the bottles were opened. The bacterial content of the water was estimated to be 2,000,000 per cubic centimeter. All the other conditions were as in the preceding experiment.

The death of the typhoid bacillus was rapid in the river water at this temperature, but not so rapid as in the preceding experiment at a temperature of 20° C. The specific bacillus was not found, however, after the fourth day. Its complete disappearance was shown by the comprehensive nature of the examination. The results are given in Table XII.

Experiment 18.—The water, after filtration and seeding as in the preceding experiment, was distributed by means of a sterile pipette in amounts of 5 c.c. each to sterile test-tubes. These were subjected to the temperature of the air outside the laboratory (below 0° C.). In the course of a few minutes the water in the tubes was frozen, and remained so except when melted for examination. Sterile stoppers of cotton were used, admitting free access of air. Light was excluded.

One tube was taken daily and the ice melted by immersing the tube for a few minutes in a water-bath heated to 35° C. One c.c. of the water was then plated in neutral agar, and the typhoid organism sought by the methods already described. It was found that even at temperatures below 0° C. the typhoid bacillus dies rapidly in the filtered water of the river, but two organisms being found on the first day and not one later. Its complete disappearance is shown by the failure to find the specific organism in plate cultures of all the water remaining in the tubes, which contained at the beginning several thousand typhoid organisms.

TABLE XII.

Day of Experiment	Amount of Water Plated	No. of Typhoid Bacilli on Plates	Estimated No. in Bottle	B. typhosus + = present 0 = absent
Seeding	1 c.c.	1,080	108,000	+
1	1	408	40,392	+
2	3	260	8,516	+
3	5	120	2,280	+
4	5	24	432	+
5	5	0	0	0
6	5	0	0	0
7	5	0	0	0
8	5	0	0	0
9	5	0	0	0
10	60	0	0	0

Experiment 19.—To 800 c.c. of the water of the Chicago River, taken at the Ashland Avenue bridge (estimated bacterial content 2,000,000 per cubic centimeter), was added a large loopful of twenty-four-hour agar culture of B. typhosus strain "x," received from Professor Jordan. A parchment sac, containing the infected water, was placed in the river at the Ashland Avenue bridge, being placed for protection in a box to which the river water had free

access, but which excluded the light. The temperature of the surrounding water was found to vary from 12° to 14° C. There was free access of air through the cotton stopper in the glass tube of the capsule.

Samples of the water were taken daily, plate cultures made in neutral agar, and, after incubation for twenty-four to forty-eight hours at 39° C., the typhoid colonies and those resembling them were subcultured in Hiss tubes, and the identity of the organisms was established by the usual cultural and agglutination tests. The typhoid bacillus was recovered with comparative ease on the first and second days, but with difficulty on the third. On the fourth day the sac was found to have a small hole in it, and the experiment was discontinued.

Experiments 20 and 21 were repetitions of Experiment 19. The typhoid bacillus was recovered up to the second and third days respectively, but not later, the experiment being continued for ten days in each case.

Experiment 22.—Race "O" from Parke, Davis & Co. was used in seeding the river water in this experiment. The estimated bacterial content before seeding was estimated to be 80,000, and the seeding to be 20,000 per cubic centimeter. The temperature of the water surrounding the parchment sac was taken daily and found to agree with that of the water in the sac, varying from 8° to 4° C. The other conditions of the experiment were as in the preceding three.

Plate cultures were made in neutral agar, using a seeding of about 1/100 c.c. The typhoid-like colonies were subcultured in Hiss tubes, and their identity was established by culture methods and the Widal test. To obviate the objections which might be made on account of the very small amounts of water used in seeding, a large number of plates were prepared, and use was made of the special method of Schepilewsky.

The typhoid organism was found with ease on the first day, and with difficulty on the second and third, after which it could not be found. Use of a special method on the fourth, seventh, and tenth days gave negative results.

Experiment 23.—This was designed as a control experiment to determine the fate of the typhoid organisms when placed under the influence of the polluted river water, but not in direct contact with the bacteria thereof. To 800 c.c. of tap water (Chicago supply), sterilized by heating for thirty minutes in the autoclave at a temperature of 120° C., was added a loopful of a twenty-four-hour culture of the typhoid organisms, as in the preceding experiments, the seeding being estimated to be about 1,500,000 per cubic centimeter. The infected water was then introduced into the parchment sac, which was subjected to the same conditions as in Experiment 19.

The typhoid bacillus was found on the first and second days, but not later.

Experiment 24.—One twenty-fifth of a small loopful of a forty-eight-hour culture of the typhoid organism (Race "O," Parke, Davis & Co.) was added to 500 c.c. of the river water, which was estimated at the time of seeding to contain 640,000 water and sewer organisms per cubic centimeter. After agitating to insure uniform seeding, the infected water was distributed to celloidin capsules in amounts of 20 c.c. each. These sacs were sealed and immersed in the water of the Chicago River at the Ashland Avenue bridge. The temperature of the water ranged from 1° to 4° C. The capsules were inclosed in

a box, to which the water had free access, but which excluded light. This protected them from ice.

Search was made as in Experiment 22, already described, for the typhoid bacillus, with the result that the organism was recovered with ease immediately after seeding, and twenty-four hours later, but not thereafter. On the fifth and the ninth days all the water in a capsule was taken and antiserum added in the proportion of 1:100. This mixture was placed in a sterile burette, and after sedimentation for twenty-four hours the lowest portion was drawn off and plated. The typhoid-like colonies were tested in the usual manner, with absolutely negative results.

Experiment 25.—In this the river water was filtered, and the celloidin sac containing it was suspended in a glass jar in about 400 c.c. of the river water, which was estimated to contain 500,000 organisms per cubic centimeter. The typhoid bacilli were thus subjected, not only to the action of the toxic agent in the filtered water of the sac, but also to that produced in the unfiltered water surrounding it. The results are presented in Table XIII:

TABLE XIII.

Day of Experiment	No. of Typhoid Bacilli per c.c.	Bacillus typhosus + = present 0 = absent
Seeding	572	+
1	330	+
2	110	+
3	49	+
4	15	+
5	0	0
6	0	0
7	0	0
8	0	0
9	0	0
10	0	0

Experiment 26.—This differed from the preceding experiment only in that normal salt solution, sterilized as in Experiment 23, was used, and the water in the glass jar in which the celloidin sac containing the infected salt solution was suspended was also rendered germ-free by filtration through a sterile unglazed porcelain filter. The typhoid bacilli were thus subjected to the action of the toxic agents in the water passing through the walls of the celloidin sac. The temperature was about 20° C. The disappearance of the

TABLE XIV.

Day of Experiment	No. of Colonies of Typhoid Bacilli	Bacillus typhosus + = present 0 = absent
Seeding	900	+
1	860	+
2	2	+
3	0	0
4	0*(infected)	0
5	0**	0
6-10	0	0

* A few saprophytes appeared on the plates.

** The cultures showed an overgrowth of saprophytes.

typhoid bacillus was sufficiently rapid to justify the conclusion that the toxic agent passed rapidly through the walls of the celloidin sac. The results appear in Table XIV.

In addition to the above experiments with the water of Lake Michigan and the Chicago River, three experiments were conducted to determine the longevity of *B. dysenteriae* Shiga in these waters.

Experiment 27.—To 500 c.c. of raw (unheated) water of Lake Michigan, taken from the tap in Dr. Zeit's laboratory and estimated to contain 200 organisms per cubic centimeter, was added 1 c.c. of 1:100 dilution in sterile salt solution of a twenty-four-hour agar culture of *B. dysenteriae* Shiga, Race "Ridgely."¹ This was estimated by culture methods to give a seeding of approximately 22,000 bacilli per cubic centimeter. After agitation to insure even seeding, the water was distributed to the celloidin sacs in amounts of 20 c.c. each, by means of a sterile pipette.

Cultures were made daily in agar, and the dysentery organism was isolated by the method of Flexner. Colonies which appeared in the second twenty-four hours were subcultured on slanted glucose litmus agar. The identity of the *B. dysenteriae* was established by culture methods and by means of its reaction with antidyenteric serum.

Large numbers of the dysentery organism were recovered on the first day, a few on the second, and none at all on the third day. Later search showed their entire disappearance. Special methods (agglutination with antidyenteric serum in a manner similar to the method of Schepilewsky for the isolation of the typhoid bacillus) gave negative results on the fifth, seventh, and tenth days.

Experiment 28.—Eight hundred c.c. of the water of the Chicago River were seeded with a small loopful of *B. dysenteriae* Shiga, Race K-72², giving an estimated seeding of about 700,000 per cubic centimeter. The germ content of the water before seeding was estimated to be about 80,000 per cubic centimeter. After agitation the infected water was placed in a parchment sac and suspended in the Chicago River at Ashland bridge, as in Experiment 19.

The method of search for the dysentery bacillus and the result of the experiment were the same as in the preceding one, except that in this case two colonies of the dysentery organism were recovered on the third day.

Experiment 29.—Raw water of the Chicago River, estimated to contain 640,000 water and sewerage organisms per cubic centimeter, was used in this experiment. Five hundred c.c. were seeded with 1:100 of a small loopful of a twenty-four-hour culture of the dysentery bacillus, Race "Ridgely" being used, giving a seeding of 22,000 per cubic centimeter. The infected water was distributed to celloidin capsules, which were sealed and immersed in the river as in the other experiments.

¹A subculture of a dysentery organism isolated from a case of "summer diarrhea" at the Thomas Wilson Sanitarium, Mount Wilson, Baltimore county, Md., during the summer of 1903. The type is now distinguished as the mannite-fractor form of the dysentery organism.

²A subculture of a culture of the dysentery bacillus kept at the pathological laboratory of Johns Hopkins University, said to be a subculture of the original organism isolated by Shiga from a case of epidemic dysentery in Japan. This organism is now distinguished as the non mannite-fractor type of the dysentery organism.

Search was made for the dysentery organism as in the two preceding experiments, the bacillus being recovered on the first day but not later. Special methods were used on the seventh and ninth days, without positive results.

TABULATED SUMMARY OF EXPERIMENTS.

TABLE XV.

A. LONGEVITY OF BACILLUS TYPHOSUS IN LAKE MICHIGAN TAP WATER.

No. of Experiment	Container	Kind of Water in Container	No. of Saprophytes per 1 c.c.	No. of Typhoid Bacilli per 1 c.c.	No. of Days Examined	Last Day on Which Typhoid Bacilli Were Found
1.....	Glass recept.	Raw	85	1,000,000	14	6
2.....	"	Raw	180	4 loops full to 1,000 c.c.	12	7
3.....	"	Raw with excreta	5,800,000	4 loops full to 1,000 c.c.	8	Immediate plating only
4.....	"	Sterilized	0	1,500,000	40	25
5.....	"	Filtered	0	500	10	4
6.....	Parchment sac suspended in flowing tap water	Raw	2,000	540,000	10	7
7.....	"	Raw	200-2,000	450,000	15	5
8.....	"	Raw	85	1,000,000	14	8
9.....	"	Sac suspended in air	85	1,000,000	14	5
10.....	"	Raw	180	3,000,000	9	6
11.....	"	Raw	180	2,300,000	10	7
12.....	"	Sterilized	0	1,000,000	15	15
13.....	Bottle, 4-oz.	Sterile salt solution 0.6%	0	1,080	10	10
14.....	Celloidin sacs suspended in flowing tap water	Raw	68	1,000,000	14	7
15.....	"	Sterilized	0	1,000,000	15	15

B. LONGEVITY OF BACILLUS TYPHOSUS IN CHICAGO RIVER WATER.

No. of Experiment	Container	Kind of Water in Container	No. of Saprophytes per 1 c.c.	No. of Typhoid Bacilli per 1 c.c.	No. of Days Examined	Last Day on Which Typhoid Bacilli Were Found
16.....	Bottle	Filtered 20° C.	1,500,000 before filt.	500	10	1
17.....	Bottle	Filt. 1-8 C.	2,000,000 before filt.	1,080	10	4
18.....	Test tubes	Filtered, kept frozen	1,000,000 before filt.	600	10	1
19.....	Parchm. sac susp. in river	Raw	2,000,000	2,000,000	4	3
20.....	"	Raw	2,500,000	1,500,000	10	2
21.....	"	Raw	2,500,000	2,000,000	10	3
22.....	"	Raw	80,000	20,000	10	3
23.....	"	Sterilized	0	1,500,000	10	2
24.....	Celloidin sacs susp. in river	Raw	640,000	11,000	10	1
25.....	"	Filtered	0	572	10	4
26.....	"	Sterilized salt solution 0.6%	0	900	10	2

C. LONGEVITY OF DYSENTERY BACILLI IN LAKE MICHIGAN TAP WATER AND CHICAGO RIVER.

No. of Experiment	Container	Kind of Water in Container	No. of Saprophytes per 1 c.c.	No. of Shiga Bacilli per 1 c.c.	No. of Days Examined	Last Day on Which Dysentery Bacilli Were Found
27.....	Celloidin sacs suspended in running tap water	Tap water	1,000,000	22,000	10	2
28.....	Parchm. sac susp. in river water	Raw river water	80,000	700,000	10	3
29.....	Celloidin sacs susp. in river water	Raw river water	640,000	22,000	10	1

EXPERIMENTS CONDUCTED IN THE CHICAGO DRAINAGE CANAL (E. O. J.).

The data of the more representative individual experiments are presented in tabular form, and the results of all the experiments are summarized. Some general statements regarding method of experimentation must first be made.

A suspension in sterile water of twenty-four-hour-old agar growths was used for inoculating the sacs, and in each case a numerical determination was made to ascertain the number of bacilli present in one cubic centimeter of the contents of the sacs, immediately after inoculation. The character of the sewage into which the bacteria were introduced was also determined bacterially, and in some cases chemically also. The composition of the fluid in the Chicago Drainage Canal is that of a dilute sewage, as shown by the following analyses:

	APRIL-JUNE, 1900	
	At Mouth of Canal (Kedzie Ave.)—Average of 9 Weekly Determinations	Bear Trap Dam at Lockport—Average of 11 Weekly Determinations
Total residue on evaporation.....	196
Dissolved residue on evaporation.....	183
Chlorin.....	12.7
Oxygen consumed (total).....	6.27
Oxygen consumed (dissolved).....	4.97
Nitrogen as free ammonia.....	1.31	1.33
Nitrogen as albuminoid ammonia.....	499.499	0.347
Bacteria per cubic centimeter.....	1,332,000	1,167,000

The amount of water flowing over the Bear Trap Dam at Lockport ranged from 250,000 to 290,000 cubic feet per minute, and averaged about 275,000 (October 1 to November 9, 1903).

The following analyses were made during the conduct of the experiments:

	DRAINAGE CANAL AT ROBEY ST.	DRAINAGE CANAL, BEAR TRAP DAM, LOCKPORT	
	Oct. 9, 1903	Oct. 12, 1903	Oct. 14, 1903
Total nitrogen (Kjeldahl).....	1.539	2.254	1.448
Nitrogen as free ammonia.....	0.839	1.174	1.302
Chlorin.....	13.3	12.0	12.0

The changes that occur in the water in the sacs (uninfected) are to a considerable extent independent of the composition of the canal water in which the sacs are suspended. The nature of these changes is shown in the following instances:

	A Comp. of Canal Water (Lockport)	B Comp. of Water in Cont. Sacs Filled 3 Days Prev. with Canal Water, (A)	C Initial Comp. of Canal Water	D (C), after Re- maining in Sacs 12 Days
Total N. (Kjeldahl)....	2.254	1.72	1.539	1.809
Albuminoid ammonia...	0.476	0.358	0.502	0.548
Free ammonia.....	1.174	1.484	0.8388	0.524
Oxygen consumed....	104.0	57.0	88.0	52.6
Chlorin.....	12.0	12.0	13.3	9.91

In the early experiments the numbers of typhoid bacteria introduced ranged from 500 to about 15,000 per cubic centimeter, but in the later experiments much larger numbers of bacteria were introduced. In one case as many as 857,000 per cubic centimeter were inoculated into the contents of a sac. Before beginning the experiments it was anticipated that typhoid bacilli would be found in considerable numbers on the third and fourth days, and consequently no examination of the contents of the sacs was made until after several days had elapsed. But the negative outcome of these early experiments showed that the death of the typhoid bacilli took place sooner than had been conjectured; and in the later tests it was found that the greatest mortality occurred during

the first forty-eight hours. The samples of water from the infected sacs were collected with sterilized 50 c.c. pipettes, transferred to sterilized collecting bottles, and brought immediately to the laboratory, where they were plated in the various media. It was found that the best results in differentiation were obtained when the plates were incubated twenty-four to forty-eight hours.

The colonies were usually examined after twenty-four hours, a selection made, and then the plate was returned to the incubator, and a second examination made twenty-four hours later. Except in the case of those samples removed from the sacs within the first few hours after inoculation, when the number of typhoid colonies was very large, an attempt was always made to pick off all typhoid-like colonies developing on each plate. Within three or four days after the inoculation of the sac, however, the number of typhoid-like colonies always diminished so greatly that colonies bearing a more or less remote resemblance to typhoid colonies had to be taken for examination. The colonies thus selected as showing the closest resemblance to typhoid colonies were transferred to tubes of glucose agar, and these tubes were incubated for forty-eight hours. Those in which gas production occurred were at once discarded, and from the tubes retained inoculations were made into milk.

A forty-eight-hour growth in milk at 37° C. eliminated a considerable proportion of those cultures that passed the glucose agar test. Those that gave a typhoid-like growth in milk were inoculated into dextrose litmus broth and into gelatin—a procedure which effected some further reduction in the number of typhoid-like colonies, although not nearly so many were thrown out as in the glucose agar and the milk tests.

Parallel cultures were made in each medium with the strains of typhoid bacilli used in the experiment, and only those cultures showing wide and unmistakable divergence from the parent stock were rejected.

Finally, the bacteria that passed all the cultural tests were examined in regard to their agglutinability, a serum (from the rabbit) being used which agglutinated the homologous strain in

a 1:1,000 dilution. No instance was found where a bacillus, that had passed all the cultural tests enumerated above, failed to agglutinate in an appropriate dilution.

Many colonies that were picked off because they presented a typhoid-like appearance on the plates afterward proved not to be *Bacillus typhosus*, but were found to belong to the fluorescent group of bacteria. By far the larger number of the organisms that passed the glucose agar test and were afterward rejected belonged to this class.

It was considered important to determine to what extent the lodgment of bacteria on the walls of the sacs was responsible for the disappearance of bacteria from the water in these sacs. Accordingly several sacs had the walls carefully swabbed with a pledglet of sterile cotton on the end of a rod, and samples of the contents of these sacs were taken before and after the swabbing. The counts showed in some cases a slight increase in numbers, indicating that some bacteria had become attached to the walls of the sacs and were dislodged by the swabbing process, but in no case was such increase very marked.

One of these comparisons of the colony count before and after swabbing is given in the following table:

TABLE XVI.

DRAINAGE CANAL AT LOCKPORT—COMPARISON OF SAC L₁ BEFORE AND AFTER SWABBING.

SAC NO.	TIME AFTER INOCULA- TION	NO. OF COLONIES PER C.C.		COLONIES FISHED	TYPHOID COLONIES FOUND
		Medium	48 hr. count		
L ₁	3 days	Drigalski	3	8	0
		Horrocks	36	4	0
		Hiss	232	8	0
L ₁ After swabbing	3 days	Drigalski	11	10	0
		Horrocks	28	20	0
		Hiss	285	10	0

A tabulated summary of typical experiments conducted in the manner above described is contained in the following tables: (XVII–XXIII).

TABLE XVII.
DRAINAGE CANAL AT LOCKPORT.

Initial colony count sewage bacteria	- - - - -	110,000
Initial colon bacilli	- - - - -	4,000-6,000
Initial streptococci	- - - - -	1,000

B. typhosus, Strain (x), was added to this sewage in such numbers that each sac contained approximately 12,000 typhoid bacilli per cubic centimeter.

Time after Inoculation	Sac No.	Amount Plated in c.c.	Medium	Colonies on Plate	Colonies Fished	Typhoid Colonies Found
1 day.....	B ₁	...	Drigalski	...	40	0
1 day.....	B ₁	...	Horrocks	...	20	
2 days.....	B ₂	...	Drigalski	...	40	0
2 days.....	B ₂	...	Horrocks	...	30	
3 days.....	"	1.0	Drigalski	...	4	0
3 days.....	"	0.1	Drigalski	...	3	
3 days.....	"	1.0	Horrocks	...	2	
3 days.....	"	0.1	Horrocks	...	3	
3 days.....	"	1.0	MacConkey	...	7	0
5 days.....	"	1.0	Drigalski	86	8	
5 days.....	"	1.0	Horrocks	50	8	
5 days.....	"	1.0	MacConkey	166	8	
7 days.....	"	1.0	Drigalski	120	25	0
7 days.....	"	1.0	Horrocks	170	20	
9 days.....	"	1.0	Neutral agar	...	20	0
9 days.....	"	1.0	Drigalski	...	12	
9 days.....	"	1.0	Horrocks	...	7	
9 days.....	"	1.0	MacConkey	...	10	

During the conduct of this experiment, October 5-14, the temperature of the air ranged from 14° to 19° C., and of the water, from 14.5° to 17° C.

TABLE XVIII.
DRAINAGE CANAL AT LOCKPORT.

Initial colony count sewage bacteria	- - - - -	110,000
Initial colon bacilli	- - - - -	4,000-6,000
Initial Streptococci	- - - - -	1,000

B. typhosus, Strain (x), was added to this sewage in such numbers that each sac contained approximately 600 typhoid bacilli per cubic centimeter.

Time after Inoculation	Sac No.	Amount Plated in c.c.	Medium	Colonies on Plate	Colonies Fished	Typhoid Colonies Found
1 day.....	C ₁	...	Drigalski	...	16	0
1 day.....	C ₁	...	Horrocks	...	4	
2 days.....	C ₂	...	Drigalski	...	20	0
2 days.....	C ₂	...	Horrocks	...	10	
3 days.....	"	1.0	Drigalski	220	20	0
3 days.....	"	1.0	Horrocks	130	10	
3 days.....	"	1.0	MacConkey	270	10	
3 days.....	"	1.0	Drigalski	...	8	
5 days.....	"	1.0	Horrocks	...	8	0
5 days.....	"	1.0	MacConkey	...	8	
7 days.....	"	1.0	Drigalski	...	4	0
7 days.....	"	1.0	Horrocks	...	5	
9 days.....	"	1.0	Drigalski	...	4	0
9 days.....	"	1.0	Horrocks	...	1	
9 days.....	"	1.0	MacConkey	...		

During the conduct of this experiment, October 5-14, the temperature of the air ranged from 14° to 19° C., and of the water, from 14.5° to 17° C.

TABLE XIX.

DRAINAGE CANAL AT LOCKPORT.

Initial colony count sewage bacteria	- - - - -	110,000
Initial colon bacilli	- - - - -	4,000-6,000
Initial streptococci	- - - - -	1,000

B. typhosus, Strain (y), was added to this sewage in such numbers that each sac contained approximately 180 typhoid bacilli per cubic centimeter. The bacilli were added in 5 c.c. of urine from a typhoid patient.

Time after Inoculation	Sac No.	Amount Plated in c.c.	Medium	Colonies on Plate	Colonies Fished	Typhoid Colonies Found
1 day.....	D ₁	1.0	Drigalski	...	10 / 13	0
1 day.....	"	1.0	Horrocks	...	3 /	
3 days.....	"	1.0	Drigalski	...	5 /	0
3 days.....	"	1.0	Horrocks	...	8 / 20	
3 days.....	"	1.0	MacConkey	...	7 /	0
5 days.....	"	1.0	Drigalski	...	6 / 12	
5 days.....	"	1.0	MacConkey	...	6 /	0
7 days.....	"	1.0	Drigalski	...	8 /	
7 days.....	"	1.0	Horrocks	...	6 / 20	0
7 days.....	"	1.0	MacConkey	...	6 /	

During the conduct of this experiment, October 5-14, the temperature of the air ranged from 14° to 19° C., and of the water, from 14.5° to 17° C.

TABLE XX.

DRAINAGE CANAL AT LOCKPORT.

Initial colon bacilli	- - - - -	1,000
Initial streptococci	- - - - -	2,000

B. typhosus, Strain (y), was added to this sewage in such numbers that each sac contained approximately 360,000 typhoid bacilli per cubic centimeter.

Time after Inoculation	Sac No.	Amount Plated in c.c.	Medium	Colonies on Plate	Colonies Fished	Typhoid Colonies Found
10 min.	O ₁	0.001	Drigalski	45	14 /	13 /
10 min.	"	0.001	Horrocks	160	18 / 68	17 / 63
10 min.	"	0.001	Hiss	230	36 /	33 /
2 days.....	"	0.1	Drigalski	410	15 /	0
2 days.....	"	0.1	Hiss	5,700	25 / 65	
2 days.....	"	0.01	Hiss	...	25 /	0
3 days.....	"	1.0	Drigalski	268	10 /	
3 days.....	"	0.1	Drigalski	...	10 /	0
3 days.....	"	1.0	MacConkey	270	10 / 63	
3 days.....	"	0.1	MacConkey	...	10 /	0
3 days.....	"	1.0	Horrocks	250	13 /	
3 days.....	"	0.1	Neutral agar	5,840	10 /	0
5 days.....	O ₂	0.1	Drigalski	...	3 /	
5 days.....	"	1.0	Drigalski	...	14 /	0
5 days.....	"	1.0	MacConkey	...	21 / 46	
5 days.....	"	0.1	Hiss	...	2 /	0
5 days.....	"	0.1	Neutral agar	...	6 /	
7 days.....	O ₁	1.0	MacConkey	126	8 / 19	0
7 days.....	"	1.0	Hiss	360	11 /	0
7 days.....	O ₂	1.0	MacConkey	32	5 / 23	
7 days.....	"	0.1	Hiss	370	18 /	0
10 days.....	"	1.0	Hiss	85	85	0

During the conduct of this experiment, October 17-27, the temperature of the air ranged from 19° to 17.5° C., and of the water, from 10° to 14.5° C.

TABLE XXI.

DRAINAGE CANAL AT ROBEY STREET.

Initial colony count sewage bacteria240,000

Initial colon bacilli..... 1,000

B. typhosus, Strain (z), was added to this sewage in such numbers that each sac contained approximately 285,000 typhoid bacilli per cubic centimeter.

Time after Inoculation	Sac No.	Amount Plated in c.c.	Medium	Colonies on Plate	Colonies Fished	Typhoid Colonies Found
1 hour.....	P ₁	0.001	Hiss	430	25	20
1 hour.....	"	0.001	Drigalski	80	6	4
1 hour.....	"	0.001	Drigalski	3 49	2 41
1 hour.....	"	0.001	MacConkey	210	15	15
20 hours.....	"	0.001	Hiss	440	37	13
20 hours.....	"	0.001	Neutral agar	610	4	1
20 hours.....	"	0.001	Drigalski	15	6 54	2 21
20 hours.....	"	0.01	MacConkey	1,780	7	5
28 hours.....	"	0.01	Hiss	1,350	33	24
28 hours.....	"	0.001	Hiss	4	1
28 hours.....	"	0.01	Drigalski	60	3 61	0 46
28 hours.....	"	0.01	MacConkey	430	21	21
2 days.....	"	0.01	Hiss	173	59	1
2 days.....	"	0.01	MacConkey ¹	3 69	0 1
2 days.....	"	0.1	MacConkey	108	7	0
3 days.....	"	0.01	Hiss	182	6 31	0
3 days.....	"	0.1	Hiss	15 21	0
4 days.....	"	0.1	Drigalski ²	45	6	0
5 days.....	"	0.1	Hiss	105	10 16	0
5 days.....	"	1.0	Drigalski	32	32 32	0
6 days.....	"	0.1	Hiss	1,344	28 36	0
7 days.....	"	0.1	Hiss	1,130	8	0
7 days.....	"	1.0	Drigalski	160	31 35	0
8 days.....	"	0.1	Hiss	440	4	0
8 days.....	"	1.0	MacConkey	120	0

During the conduct of this experiment, October 25 to November 4, the temperature of the air ranged from 6° to 13° C., and of the water, from 9.5° to 12° C.

TABLE XXII.

DRAINAGE CANAL AT ROBEY STREET.

Initial colony count sewage bacteria240,000

Initial colon bacilli..... 1,000

B. typhosus, Strain (z), was added to this sewage in such numbers that each sac contained approximately 285,000 typhoid bacilli per cubic centimeter.

Time after Inoculation	Sac No.	Amount Plated in c.c.	Medium	Colonies on Plate	Colonies Fished	Typhoid Colonies Found
3 days.....	P ₂	0.01	Hiss	1,130	14	0
3 days.....	"	0.1	Drigalski ³	229	3 19	0
3 days.....	"	0.01	MacConkey	35	2	0
4 days.....	"	0.1	Hiss	6,120	13 18	0
4 days.....	"	1.0	MacConkey ⁴	5	0
5 days.....	"	0.1	Hiss	1,880	23 33	0
5 days.....	"	1.0	MacConkey	97	10	0
6 days.....	"	0.1	Hiss	1,540	15 23	0
6 days.....	"	1.0	Drigalski	83	8	0
7 days.....	"	0.1	Hiss	1,296	11	0
7 days.....	"	0.1	Neutral agar	670	5 18	0
7 days.....	"	1.0	Drigalski	44	2	0
8 days.....	"	0.1	Hiss	1,890	18 41	0
9 days.....	"	0.1	Hiss	1,960	8	0
9 days.....	"	0.1	Neutral agar	1,460	13 37	0
9 days.....	"	1.0	MacConkey	140	20	0
10 days.....	"	0.1	Hiss ⁵	1,820	24	0
10 days.....	"	1.0	MacConkey	86	13	3

During the conduct of this experiment, October 25 to November 4, the temperature of the air ranged from 6° to 13° C., and of the water, from 9.5° to 12° C.

¹ All typhoid-like colonies on MacConkey fished.² All typhoid-like colonies on Drigalski fished.³ All typhoid-like colonies on MacConkey and Drigalski fished.⁴ All typhoid-like colonies on MacConkey fished.⁵ All surface typhoid-like colonies on Hiss and MacConkey fished.

TABLE XXIII.
DRAINAGE CANAL AT ROBEY STREET.

Initial colony count sewage bacteria.....240,000
Initial colon bacilli.....1,000

B. typhosus, Strain (y), was added to this sewage in such numbers that each sac contained approximately 857,000 typhoid bacilli per cubic centimeter. The bacilli were added in 50 c.c. of urine from a typhoid patient.

Time after Inoculation	Sac No.	Amount Plated in c.c.	Medium	Colonies on Plate	Colonies Fished	Typhoid Colonies Found
20 hours.....	N ₁	0.001	Hiss	730	17	5
20 hours.....	"	0.001	Neutral agar	1,040	2	0
20 hours.....	"	0.001	Drigalski	118	7	0
20 hours.....	"	0.01	Drigalski	15	6
20 hours.....	"	0.01	MacConkey	960	9	9
28 hours.....	"	0.01	Hiss	540	32	23
28 hours.....	"	0.01	Drigalski	380	15	10
28 hours.....	"	0.01	MacConkey	392	10	43
2 days.....	"	0.01	Hiss	23	3
2 days.....	"	0.1	Drigalski	14	0
2 days.....	"	0.01	MacConkey ¹	5	4
2 days.....	"	0.1	MacConkey	10	2
3 days.....	"	0.01	Hiss ²	1,072	36	0
3 days.....	"	0.1	Drigalski	4,080	8	56
3 days.....	"	0.01	MacConkey	520	12	0
4 days.....	"	0.01	Hiss ³	2,040	25	35
4 days.....	"	0.1	Hiss	10	0
5 days.....	"	0.1	Hiss	7,200	16	23
5 days.....	"	1.0	Drigalski	220	7	0
6 days.....	"	0.1	Neutral agar	1,490	4	10
6 days.....	"	1.0	Drigalski	180	6	0
7 days.....	"	0.1	Hiss	1,890	25	25
7 days.....	"	0.1	Hiss	1,300	23	23
8 days.....	"	0.1	Hiss	17,400	13	32
9 days.....	"	1.0	MacConkey	214	19	0

During the conduct of this experiment, October 25 to November 4, the temperature of the air ranged from 6° to 13° C., and of the water, from 9.5° to 12° C.

TABLE XXIV.
DRAINAGE CANAL AT ROBEY STREET—SUMMARY.

SAC NO.	NO TYPH. BAC. IN-TROD.	1 HOUR		20 HOURS		28 HOURS		2 DAYS	
		No. Col. Taken in	No. Typh. Found	No. Col. Taken in	No. Typh. Found	No. Col. Taken in	No. Typh. Found	No. Col. Taken in	No. Typh. Found
P ₁	285,000	c.c. 0.0001... 3	2	c.c. 0.001... 47	16	c.c. 0.001... 4	1	c.c. 0.01... 62	1
P ₂	285,000	0.001... 46	39	0.01... 7	5	0.01... 57	45	1.0... 7	0
N ₁	857,000	0.001... 26	5	0.01... 57	43	0.01... 28	7
N ₂	857,000	0.01... 24	15	0.1... 24	2
F ₁	11,500
F ₂	11,500
F ₃	11,500
G ₁	670	0.1... 25	0
G ₂	670	1.0... 10	0
H ₁	18,000
H ₂	18,000
H ₃	18,000
I ₁	900	0.1... 25	0
R ₁	25,000	1.0... 10	0
R ₂	25,000	0.1... 11	0
S ₁	16,800	1.0... 9	0
Total		49	41	104	41	118	89	221	10

¹ All typhoid-like colonies on MacConkey (0.01) fished.

² No surface typhoid-like colonies on Hiss (0.01) and Drigalski.

³ All surface typhoid-like colonies on Hiss fished.

TABLE XXIV.—Continued.

SAC No.	No. TYPH. BAC. IN- TROD.	3 DAYS		4 DAYS		5 DAYS		6 DAYS	
		No. Col. Taken in	No. Typh. Found	No. Col. Taken in	No. Typh. Found	No. Col. Taken in	No. Typh. Found	No. Col. Taken in	No. Typh. Found
P ₁	285,000	c.c. 0.01.....25	0	c.c. 0.1.....15	0	c.c. 0.1.....10	0	c.c. 0.1.....32	0
		0.1.....6	0	1.0.....6	0	1.0.....6	0		
P ₂	285,000	0.01.....16	0	0.1.....13	0	0.1.....23	0		..
		0.01.....3	0	1.0.....5	0	1.0.....10	0		
N ₁	857,000	0.01.....48	0	0.01.....23	0	0.1.....16	0	0.1.....4	0
		0.1.....8	0	0.1.....10	0	1.0.....7	0	1.0.....6	0
N ₂	857,000	0.01.....6	0	0.1.....15	0	1.0.....12	0
				0.1.....8	0	1.0.....14	0	1.0.....20	0
F ₁	11,500	1.0.....25	0
F ₂	11,500
F ₃	11,500
G ₁	670	1.0.....15	0
G ₂	670
H ₁	18,000	1.0.....50	0	1.0.....19	0
H ₂	18,000
H ₃	18,000	1.0.....17	0
I ₁	900	1.0.....15	0
R ₁	25,000	0
R ₂	25,000	0.001.....19	0	0.01.....12	0
S ₁	16,800	0.0001.....4	0	0.01.....14	0
		0.001.....13	0
Total		142	0	193	0	127	0	133	0

TABLE XXIV.—Continued.

SAC No.	No. TYPH. BAC. IN- TROD.	7 DAYS		8 DAYS		9 DAYS		10 DAYS	
		No. Col. Taken in	No. Typh. Found	No. Col. Taken in	No. Typh. Found	No. Col. Taken in	No. Typh. Found	No. Col. Taken in	No. Typh. Found
P ₁	285,000	c.c. 0.1.....28	0	c.c. 0.1.....31	0	c.c.	c.c.
		1.0.....8	0	1.0.....4	0
P ₂	285,000	0.1.....16	0	0.1.....18	0	0.1.....21	0	0.1.....24	0
		1.0.....2	0	1.0.....20	0	1.0.....13	3
N ₁	857,000	1.0.....25	0	1.0.....23	0	0.1.....13	0
		1.0.....19	0
N ₂	857,000	0.1.....8	0	0.1.....35	0
				1.0.....21	0	1.0.....24	0
F ₁	11,500
F ₂	11,500	1.0.....15	0
F ₃	11,500
G ₁	670
G ₂	670	1.0.....23	0
H ₁	18,000	1.0.....22	0
H ₂	18,000
H ₃	18,000
I ₁	900
R ₁	25,000
R ₂	25,000
S ₁	16,800
Total		139	0	76	0	102	0	96	3

TABLE XXIV.—Continued.

SAC. No.	No. TYPH. BAC. IN- TROD.	11 DAYS		14 DAYS		12 DAYS	
		No. Col. Taken in	No. Typh. Found	No. Col. Taken in	No. Typh. Found	No. Col. Taken in	No. Typh. Found
P ₁	285,000	c.c.	..	c.c.	..	c.c.	..
P ₂	285,000
N ₁	857,000
N ₂	857,000
F ₁	11,500
F ₂	11,500	1.0.....19	0	1.0.....27	0
F ₃	11,500	1.0.....41	0
G ₁	670
G ₂	670
H ₁	18,000
H ₂	18,000	1.0.....14	0	1.0.....23	0
H ₃	18,000	1.0.....34	0	0
I ₁	900
R ₁	25,000
R ₂	25,000
S ₁	16,800
Total		33	0	75	0	50	0

Total colonies taken = 1,658

Total typhoid found = 184

Total colonies taken after 2 days = 1,166

Total typhoid found after 2 days = 3

TABLE XXV.

DRAINAGE CANAL AT LOCKPORT—SUMMARY.

SAC No.	No. Typh. Bac. Intro. per c.c.	10 MINUTES		1 DAY		2 DAYS		3 DAYS		5 DAYS	
		No. Col. Taken in	No. Typh. Found	No. Col. Taken in	No. Typh. Found	No. Col. Taken in	No. Typh. Found	No. Col. Taken in	No. Typh. Found	No. Col. Taken in	No. Typh. Found
O ₁ ..	360,000	c.c.	..	c.c.	..	c.c.	..	c.c.	..	c.c.	..
		0.001....68	63	0.01....25	0	0.1....43	0	0
O ₂ ..	360,000	0.1....40	0	1.0....20	0	0.1.. 9	0
		1.0.. 35	..
B ₁ ..	12,00060	0	0
B ₂ ..	12,000	0.1.... 6	0	1.0.. 24	..
		1.0....13	0
C ₁ ..	60020	070	0	0
C ₂ ..	600	1.0....50	0	1.0.. 24	0
D ₁ ..	180	1.0....13	030	0	1.0....20	0	1.0.. 12	..
L ₁ ..	30,000	0.01.... 8	0	0
		1.0....12	0
L ₂ ..	30,000	0.01.... 2	0	0.1.. 7	0
		1.0....18	0	1.0.. 5	..
L ₃ ..	30,000	1.0.. 12	0
M ₁ ..	1,500	0.01.... 5	0
M ₂ ..	1,500	1.0.... 5	0	0.1.. 8	0
Total		68	63	93	0	165	0	202	0	136	0

TABLE XXV.—*Continued.*

Sac No.	No. Typh. Bac. Introd. per c.c.	6 DAYS		7 DAYS		8 DAYS		9 DAYS		10 DAYS	
		No. Col. Taken in	No. Typh. Found	No. Col. Taken in	No. Typh. Found	No. Col. Taken in	No. Typh. Found	No. Col. Taken in	No. Typh. Found	No. Col. Taken in	No. Typh. Found
		c.c.		c.c.		c.c.		c.c.		c.c.	
O ₁ ..	360,000	1.0.....19	0
O ₂ ..	360,000	0.1.....18	0	1.0.....85	0
				1.0.....5	0						
B ₁ ..	12,000
B ₂ ..	12,000	1.0.....45	0	1.0.....49	0
C ₁ ..	600
C ₁ ..	600	1.0.....12	0	1.0.....10	0
D ₁ ..	180	1.0.....20	0
L ₁ ..	30,000	1.0.....17	0	1.0.....24	0	1.0.....26	0
L ₂ ..	30,000
L ₃ ..	30,000
M ₁ ..	1,500
M ₂ ..	1,500
Total.....		17	0	119	0	24	0	59	0	111	0

Total colonies taken = 992

Total typhoid found = 63

Total colonies taken after day of inoculation = 924

Total typhoid found after 10 minutes = 0

The tabular résumé shows that, with one exception, no typhoid bacilli were found in plates made from any of the sacs later than two days after inoculation. The decrease in numbers seems to be most marked between twenty-four and forty-eight hours. In most instances all the typhoid-like colonies on the plate were taken, and although 1,166 colonies in all were examined, only three of these proved to be typhoid. In one case all the colonies on the plate (1 c.c.) were picked off and examined (Table XX, 10 days).

Regarding the finding of three colonies of typhoid bacilli on the tenth day after inoculation (Table XXII), it should be noted that these three colonies all appeared on a single plate, and although a number of plates from this same sac had been made during the preceding six days, no typhoid colonies whatever had been discovered. This sudden appearance of typhoid bacilli on a single plate creates the suspicion that their survival may have been due to some accidental cause, and that they had not persisted in the water of the sac during the whole period of the experiment. It seems a plausible supposition that these bacilli

may have adhered to some concrete particle in the sewage when they were freshly introduced, and that this particle dried upon the walls of the tube and remained there until accidentally washed off during the process of agitating the contents of the sac. It is, of course, possible that these three bacilli were unusually resistant individual cells which maintained their vitality in the water of the sac throughout the period covered by the experiment. This, however, seems unlikely, in view of all the other findings in the course of this investigation, and the circumstances under which these bacilli appeared indicate that their occurrence was an accidental instead of a significant one.

In addition to the sacs of sewage infected with typhoid bacilli, a number of control experiments were carried out upon uninoculated sewage (Table XXVI). The number of colonies developing on neutral agar plates, and also the number of colon bacilli and streptococci, were determined. The results obtained in this way afford an interesting confirmation of the results obtained with the typhoid-infected sacs. The number of colon bacilli remains nearly constant for about twenty-four hours, and then rapidly diminishes, falling, after five or six days, to a point far below the initial number. The streptococci, while sometimes more numerous at the outset than the colon bacilli, fall more rapidly and practically disappear within a few days after the opening of the experiment. The number of the colonies developing on agar plates shows a slight increase in the first twenty-four hours, and then a decline which is noticeably rapid and extreme.

TABLE XXVI.
CONTROLS (UNINOCULATED SEWAGE).
LOCKPORT.

No. Days in Sac	Sac No.	Colony Count Agar 57	No. B. coli in 1 c.c.	No. Streptococci in 1 c.c.
Initial.....	A ₁	470,000	4,000-6,000	1,000
3 days.....	A ₂	2,000	Less than 1,000	1,000
7 days.....	A ₂	660	50	None in 1 c.c.
9 days.....	A ₃	2,520	5	None in 10 c.c.
Initial.....	K ₁	?	1,000	2,000
3 days.....	K ₁	8,000	10	20
5 days.....	K ₂	6-9	None in 5 c.c.
6 days.....	K ₂	1,332	6-8	None in 5 c.c.
8 days.....	K ₁	11,800	7

ROBEY STREET.

No. Days in Sac	Sac No.	Colony Count Agar 37	No. B. coli in 1 c.c.	No. Streptococci in 1 c.c.
Initial.....	E ₁	380,000	4,000-6,000	40,000
2 days	E ₁	21,000	100	20
4 days	E ₁	19,500	70-90	1 in 5 c.c.
6 days	E ₁	70,200	50	None in $\frac{1}{2}$ c.c.
7 days	E ₂	17,200	90-100	None in 1-10 c.c.
11 days	E ₂	14,600	20	None in 1-5 c.c.
12 days	E ₃	75,600	4-10	None in 2-5 c.c.
14 days	E ₃	Too thick to count	6-10	
Initial.....	V ₁	240,000	800-1,000
20 hours	V ₁	327,000	700-900
28 hours	V ₁	326,000	800
2 days	V ₁	122,000	100-200
4 days	V ₁	17,000	20
5 days	V ₁	16,400	7-8
6 days	V ₁	17,800	5-6
7 days	V ₁	7,900	8
8 days	V ₁	5,980	9
9 days	V ₁	7,500	6-8

It may be concluded, therefore, that the vast majority of the typhoid bacilli introduced into the sewage of the Chicago Drainage Canal, under the conditions which prevailed during the conduct of this experiment, disappear within two days after their introduction, and that while it may be true that individual cells endowed with special powers of resistance maintain their vitality for a longer period, the outcome of this experiment shows that such an assumption finds little warrant. Furthermore, assuming that such survival of individual typhoid bacilli with rare powers of resistance did sometimes occur, there is some ground for supposing that such adaptation to a saprophytic mode of life might be associated with a lowered virulence. The facts instanced by Hankin,¹ and by Remlinger and Schneider,² regarding the occasional presence in natural waters of typhoid bacilli apparently possessed of slight virulence, or even wholly innocuous, are sufficient to demand careful consideration of this possibility.

One experiment was also made upon Lake Michigan (tap) water for the purpose of comparing the course of events in the parchment and celloidin sacs under similar conditions. Large celloidin sacs with a capacity of about 700 c.c. were prepared, and these were suspended in running water at 16° C. side by side with parchment sacs. The experiment shows that even when a small number of typhoid bacilli (3,080) were introduced into the lake

¹ *Centralbl. f. Bakt.*, 1899, 26, p. 504.² *Ann. de l'Inst. Past.*, 1897, 11, p. 55.

water, survivors could be found four days after inoculation, or twice as long as in the water of the Drainage Canal. Incidentally the experiment affords confirmatory evidence of the validity of the methods of isolation employed, since the presence of very large numbers of water bacteria (over 100,000) did not prevent the discovery of the typhoid bacilli. (*Cf.* Table XXVII, 1, 2, and 3 days.)

TABLE XXVII.

B. typhosus, Strain (y), was added to tap water, colony count 65, in such numbers that each sac contained approximately 3,080 typhoid bacilli per cubic centimeter.

PARCHMENT SAC						CELLOIDIN SAC					
Time After In-oculation	Amount Plated in c.c.	Medium	Colonies on Plate	Colonies Fished	Typhoid Colo-nies Found	Amount Plated in c.c.	Medium	Colonies on Plate	Colonies Fished	Typhoid Colo-nies Found	
1 day	0.1	Hiss	2,800	12 } 21	10 } 19	0.1	Hiss	116,000	11 } 16	3 } 6	
1 day	1.0	MacConkey	1,600	9 } 9	9 } 9	1.0	MacConkey	1,860	5 } 5	3 } 3	
2 days	0.1	Hiss	31,200	10 } 20	2 } 9	0.1	Hiss	88,000	12 } 20	1 } 2	
2 days	1.0	MacConkey	1,550	10 } 7	7 } 5	1.0	MacConkey	2,400	8 } 20	1 } 0	
3 days	0.1	Hiss	121,400	14 } 20	0 } 5	0.1	Hiss	110,400	14 } 20	0 } 0	
3 days	1.0	MacConkey	2,810	6 } 5	5 } 0	1.0	MacConkey	9,840	6 } 20	0 } 0	
4 days	0.1	Hiss	119,200	14 } 20	1 } 1	0.1	Hiss	36,800	14 } 20	1 } 1	
4 days	1.0	MacConkey	2,720	6 } 0	0 } 0	1.0	MacConkey	4,188	6 } 20	0 } 0	
5 days	0.1	Hiss	118,000	16 } 20	0 } 0	0.1	Hiss	20,100	16 } 20	0 } 0	
5 days	1.0	MacConkey	2,160	4 } 0	0 } 0	1.0	MacConkey	1,900	4 } 20	0 } 0	
6 days	0.1	Hiss	31,200	16 } 20	0 } 0	0.1	Hiss	10,440	16 } 20	0 } 0	
6 days	1.0	MacConkey	2,600	4 } 0	0 } 0	1.0	MacConkey	1,060	4 } 20	0 } 0	
7 days	0.1	Hiss	29,800	14 } 17	0 } 0	0.1	Hiss	9,200	12 } 17	0 } 0	
7 days	1.0	MacConkey	2,520	3 } 0	0 } 0	1.0	MacConkey	830	5 } 17	0 } 0	
8 days	1.0	Hiss	26,800	16 } 20	0 } 0	1.0	Hiss	3,480	16 } 20	0 } 0	
8 days	1.0	MacConkey	1,670	4 } 0	0 } 0	1.0	MacConkey	480	4 } 22	0 } 0	
9 days	1.0	Hiss	6,240	16 } 20	0 } 0	1.0	Hiss	3,160	18 } 22	0 } 0	
9 days	1.0	Drigalski	1,720	4 } 0	0 } 0	1.0	Drigalski	980	4 } 20	0 } 0	
11 days	1.0	Hiss	3,400	16 } 20	0 } 0	1.0	Hiss	520	13 } 20	0 } 0	
11 days	1.0	Drigalski	720	4 } 0	0 } 0	1.0	Drigalski	72	7 } 20	0 } 0	
12 days	1.0	Hiss	5,040	16 } 20	0 } 0	1.0	Hiss	410	16 } 20	0 } 0	
12 days	1.0	MacConkey	740	4 } 0	0 } 0	1.0	MacConkey	51	4 } 20	0 } 0	
13 days	1.0	Hiss	7,100	12 } 20	0 } 0	1.0	Hiss	2,400	0 } 20	0 } 0	
13 days	1.0	MacConkey	967	8 } 0	0 } 0	1.0	MacConkey	420	20 } 20	0 } 0	
15 days	1.0	Hiss	5,640	12 } 20	0 } 0	1.0	Hiss	550	4 } 20	0 } 0	
15 days	1.0	MacConkey	926	8 } 0	0 } 0	1.0	MacConkey	180	16 } 20	0 } 0	

LONGEVITY OF PARATYPHOID BACILLI.

In addition to the experiments that were carried out with the typhoid bacillus, one test was made of the longevity of the paratyphoid bacillus (Paratyphoid Scott) in the water of the Drainage Canal at Robey Street. The culture used was one isolated in this laboratory in June, 1903, about three months before these experiments were undertaken.¹

The methods used in the conduct of this experiment were precisely similar to those that have been described in the work upon *B. typhosus*, and the method of tabulation of the result is also similar (Tables XXVIII and XXIX). In the case of this organism a general correspondence was observed with the behavior of the typhoid bacillus, the most rapid disappearance

TABLE XXVIII.

DRAINAGE CANAL AT ROBEY STREET.

Initial colony count sewage bacteria	- - - - -	240,000
Initial colon bacilli	- - - - -	1,000

B. paratyphosus, Strain (pt), was added to this sewage in such numbers that each sac contained approximately 820,000 paratyphoid bacilli per cubic centimeter.

Time After Inoculation	Sac No.	Amount Plated in c.c.	Medium	Colonies on Plate	Colonies Fished	Typhoid Colonies Found
20 hours.....	T ₁	0.01	Hiss	4,840	40	22
20 hours.....	"	0.001	Neutral agar	550	3	1
20 hours.....	"	0.001	Drigalski	61	9	4
20 hours.....	"	0.01	Drigalski	8	2
20 hours.....	"	0.01	MacConkey	230	3	1
28 hours.....	"	0.001	Hiss ²	44	6	2
28 hours.....	"	0.01	Hiss	43	20
28 hours.....	"	0.01	Drigalski	610	2	4
28 hours.....	"	0.01	MacConkey	230	2	0
2 days.....	"	0.01	Hiss	35	2
2 days.....	"	0.01	Drigalski ³	6	1
2 days.....	"	0.1	MacConkey	8	0
3 days.....	"	0.01	Hiss	300	16	0
3 days.....	"	0.1	Hiss	7	0
3 days.....	"	0.1	Drigalski	107	10	2
4 days.....	"	0.1	Hiss	3,480	15	15
5 days.....	"	0.1	Hiss	2,720	16	16
5 days.....	"	0.1	Neutral agar	456	8	31
5 days.....	"	1.0	MacConkey	37	7	0
6 days.....	"	0.1	Hiss	1,688	13	19
6 days.....	"	1.0	Drigalski	280	6	6
7 days.....	"	0.1	Hiss	1,410	15	25
7 days.....	"	0.1	Neutral agar	1,079	10	10
8 days.....	"	0.1	Hiss	1,310	14	14
8 days.....	"	1.0	MacConkey	14	3	17

During the conduct of this experiment, October 23 to November 4, the temperature of the air ranged from 6° to 13° C., and of the water, from 9.5° to 12° C.

¹ WELLS AND SCOTT, *Jour. Infect. Dis.*, 1904, 1, p. 72.

² All typhoid-like colonies on Drigalski, MacConkey, and Hiss (0.001) fished.

³ All typhoid-like colonies on Drigalski (0.01) and MacConkey (0.1) fished.

appearing to take place within the first forty-eight hours after inoculation. Two colonies were found on the third day after inoculation, but thereafter up to the eighth day, in two sets of sacs, no paratyphoid colonies were discovered. These experiments appear to indicate that the life of this organism in the water of the Drainage Canal, although possibly slightly more extended than that of the typhoid bacillus, is still very brief.

TABLE XXIX.

DRAINAGE CANAL AT ROBEY STREET.

Initial colony count sewage bacteria - - - - - 240,000
Initial colon bacilli - - - - - 1,000

B. paratyphosus, Strain (pt), was added to this sewage in such numbers that each sac contained approximately 820,000 paratyphoid bacilli per cubic centimeter.

Time After Inoculation	Sac No.	Amount Plated in c.c.	Medium	Colonies on Plate	Colonies Fished	Paratyph. Colonies Found
4 days	T ₂	0.1	Hiss	7,380	19 $\frac{1}{2}$ 25	0
4 days	"	1.0	MacConkey	240	6 $\frac{1}{2}$ 25	0
5 days	"	0.1	Hiss	3,040	16 $\frac{1}{2}$ 24	0
5 days	"	0.1	Neutral agar	2,400	2 $\frac{1}{2}$ 24	0
5 days	"	1.0	MacConkey	83	6 $\frac{1}{2}$ 24	0
6 days	"	0.1	Hiss	2,200	6 $\frac{1}{2}$ 6	0
8 days	"	0.1	Neutral agar	860	6 $\frac{1}{2}$ 6	0

During the conduct of this experiment, October 25 to November 4, the temperature of the air ranged from 6° to 13° C., and of the water, from 9.5° to 12° C.

EXPERIMENTS CONDUCTED IN THE ILLINOIS RIVER AT AVERYVILLE, ILLINOIS (H. L. R.).

Before giving the results of the experiment made at this station, the following observations, collected on the temperature conditions of the river and the stage of water, are presented:

TABLE XXX.

DATA AS TO CONDITIONS IN RIVER (STAGE OF WATER, TEMPERATURE, AND TURBIDITY).

Date	Temperature of Water (°C)	Temperature of Air (°C—9 A. M.)	Stage (Feet above Low-Water Mark)
October 10	17.0	11.0	11.7
October 12	16.5	14.0	11.4
October 13	16.0	17.0	11.4
October 14	16.0	15.0	11.4
October 15	16.0	15.5	11.3
October 16	16.0	15.0	11.3
October 19	14.0	11.0	11.1
October 20	11.0
October 21	14.0	16.0	11.0
October 23	13.0	8.0	11.0
October 26	11.0	6.0	10.8

As the matter of temperature is of material importance in affecting the vitality of organisms, it should be kept in mind that the temperature to which the typhoid organisms were exposed in this case was materially below the mean summer heat, and would therefore favor the retention of vitality to some extent, as far as this factor is operative. Regarding the stage of the river, the data show very uniform conditions. The lowest stage noted during this summer was about eight feet above low water. At the time these experiments were conducted the turbidity in the stream was somewhat higher than usual, objects not being visible at the depth of a foot or more.

Three series of tests were made at Averyville, using fresh cultures of the three strains of the typhoid organism isolated by Professor Jordan, and designated by him as "x," "y," and "z."

In the "x" series five parchment sacs were infected; in the "y" series, an equal number; and in the "z" series, four, making in all fourteen different exposures. The "x" and "y" series were placed in the river on October 9, 1903, and "z" cultures on October 14. Samples were removed from some of the sacs at daily intervals until the end of the experiment (October 26). The first two series were therefore exposed to the influence of the river water for a period of seventeen days, and the "z" series for twelve days.

In addition to these sacs, which were first inoculated with river water and then infected with varying amounts of typhoid cultures, two other sacs filled with raw water, but uninfected with typhoid, were kept under the same conditions as controls. These were examined at intervals in order to learn the course of the bacterial changes which went on in the parchment sacs when filled with the raw water alone.

It is a well-known fact that when a small quantity of water is inclosed in a glass container, a rapid and extensive multiplication of water bacteria occurs. Just what forces are responsible for this marked proliferation is not fully understood, but the variation in experimental conditions which obtained in these experiments as to character of container rendered it especially desirable that data should be secured on this point.

Samples were therefore removed at intervals and plated on Drigalski and Conradi's medium. The results of these tests showed an increase in number of organisms per cubic centimeter, but this was only a few fold and maintained for a brief period of time. It was impossible, for lack of time, to make a study of the quantitative changes on plain agar, but doubtless it would have been greater than here reported. The results for one of these control sacs are tabulated below:

TABLE XXXI.

BACTERIAL CONTENT PER CUBIC CENTIMETER OF PARCHMENT SACS FILLED WITH RAW WATER AND IMMersed IN RIVER.

NO. DAYS' IMMERSION IN RIVER WATER	PERIOD OF INCUBATION OF CULTURES			
	1 Day	2 Days	3 Days	4 Days
1	6,000	32,000
2	18,000	68,000
5	2,700	5,500
7	20
9	90

From these data it appears that the use of the selective typhoid medium (Drigalski and Conradi) did not succeed in inhibiting entirely the growth of some of the water forms, although these indigenous species rapidly disappeared when cultivated in this medium. Attempts were made in the typhoid-infected sacs to obtain some general idea of the rate of bacterial changes. Of course, here the problem was complicated by the presence of the typhoid forms which are not greatly affected when cultivated in this culture medium. The general course of these changes was quite similar in all cases. The maximum germ content in such cases on Drigalski and Conradi's medium was found at first or within a couple of days. After this a rapid decline set in, so that, when the sacs were withdrawn from the river at the expiration of the work, they contained only a very few bacteria capable of growing on this medium.

Ratio of typhoid to indigenous water organisms.—Reference has been previously made to the question of seeding, as to the importance of knowing approximately how many typhoid organ-

isms were exposed to the water, and the effect which increased seedings have on the vitality of organisms. Under the conditions of exposure in the river, the ratio of typhoid organisms to the bacteria normally present in the river water was very much greater than could possibly occur in nature. The extremes have been previously given, but the estimated seedings for the different sacs are herewith presented in detail:

TABLE XXXII.
VARIATION IN TYPHOID SEEDINGS IN DIFFERENT SACS.

Series "x"		Series "y"		Series "z"	
Sac 1.....	1,000	Sac 6.....	1,000	Sac 12.....	540
2.....	2,000	7.....	2,000	13.....	540
3.....	4,000	8.....	4,000	14.....	5,400
4.....	10,000	9.....	10,000	15.....	10,800
5.....	20,000	10.....	20,000		

When it is remembered that the normal germ content on plain agar at blood heat of the river water at this station ranges from 60 to 500, it is at once apparent that the extent of typhoid infection is here vastly greater than would normally occur under natural conditions.

As a matter of fact, the culture plates made from the infected sacs at this station rarely showed the presence of acid colonies. When the first series of cultures were prepared, a large number of colonies developed, but these fell off rapidly. This fact greatly simplified the labor of selecting the presumptive typhoid colonies from the plates. In the later stages the colonies became more atypical for typhoid and much reduced in numbers. Under these conditions relatively large numbers were taken, so as to insure finding typhoid if present.

In making cultures from the water, sets of at least three plates were prepared with Drigalski & Conradi's medium, using a somewhat wide range in dilution (1:20 to 1:2,000 c.c.). This permitted the selection of plates having a satisfactory distribution of colonies. As the germ content declined, the amount of liquid used in seeding was increased, until, toward the end, plates were prepared containing 1 c.c. of water. Ordinarily the plates were studied the first day, the supposedly typhoid colonies being fished

and inoculated into glucose agar, then incubated. The plates were further incubated at 20° C. for another twenty-four hours and gone over again. The sugar agar cultures were then inspected, all showing gas being thrown out, and the balance sent to Madison for further study. The culture methods followed here were essentially the same as used by E. O. J. at Chicago, viz.: litmus milk, dextrose litmus broth, glucose-free broth for indol, and nutrient gelatin. Cultures that showed positive typhoid characters on these culture media were then subjected to the agglutination test, using a homologous serum of 1:500. They were also subjected to a highly potent typhoid serum secured from Parke, Davis & Co. In the subjoined tables are given the results of the various examinations, including, as far as possible, the data as to total number of colonies fished and number found to be typhoid. As the entire work of plate-culturing at Peoria devolved upon Mr. Hastings, it was impossible for him at times to compute the total colony count on each set examined, but the data collected are sufficient to show a steady and often quite rapid decline in total number of appearing colonies.

In testing these various sacs emphasis was placed in each series on the minimum and maximum seeding. If the typhoid organism could be demonstrated in these two extremes, it was, of course, highly probable that intermediate seedings would fall within these limits. In the "x" series positive results were not secured with the frequency noted in the remaining series. This was due to the fact that the parchment tubing was soaked in river water before being seeded with the typhoid culture. Under these conditions it became somewhat fouled with suspended sediment, thereby much increasing the germ content of the inclosed water for a time.

It is evident from the data submitted in Tables XXXIII-XXXV that the amount of seeding was sufficient to permit of the detection of the organism, if it was present at all. The sacs containing one-twentieth as many typhoid organisms as the sac seeded with the maximum quantity showed in both the "y" and "z" series the presence of the organism for as long a period as in any case.

It was unfortunate that a larger number of the sacs were not

tested until after the lapse of several days, but we had no expectation that the organisms introduced would die off so rapidly. It should be kept in mind that the final records as to whether the isolated cultures were true typhoid or not were not made until long after the removal of the sacs from the water. Therefore it was not possible to check up the earlier gaps in the analytical records. During these earlier days the typhoid organisms were doubtless more or less abundant, but the much larger number of tests made on the greatly reduced germ content of the sacs after the first few days, and the practical absence of the introduced organism, greatly strengthened the conclusion that the typhoid germ had lost its vitality in a relatively short period of time.

The detailed data that were collected from the examination of the various sacs of the three series are presented in the following tables:

TABLE XXXIII.

RESULTS OBTAINED FROM CULTURES MADE FROM PARCHMENT SACS AFTER VARYING PERIODS OF EXPOSURE IN RIVER WATER.

SERIES INFECTED WITH "X" CULTURE.

No. of Sacs	Dosage of Typh. Bacilli per c.c.	Time after Inoculation When Examined—Days	Total No. of Colonies on Plates Made	No. of Colonies Fished	No. of Typhoid Colonies
1.....	1,000	1	330	12	1
		3	300	9	0
		5	25	11	0
		10	125	10	0
2.....	2,000	4	100	8	0
3.....	4,000	6	...	12	0
4.....	10,000	4	50	13	0
		5	...	3	0
		7	37	8	0
5.....	20,000	1	75	6	0
		3	25	13	2
		5	12	9	0
		6	...	8	0
		7	200	7	0
		10	100	12	0
		12	540	12	0
		14	288	11	0
		17	135	15	0

The results in this first series were not as satisfactory as in the remaining series, because of the fouling of the sacs with mud before inoculation. The sacs were placed in the river water to soak up before they were infected, and some sediment from river

water settled on them. The results obtained in these series showed the presence of the typhoid organism in sacs containing the minimum and maximum amount of typhoid seeding. In the latter case, containing 20,000 typhoid bacilli per cubic centimeter, nine different examinations were made, ninety-three colonies in all being fished from the culture plates. The inoculated organism was not found subsequent to the third day. Of this number seventy-four colonies were examined after the third day, and in no case was any organism found which resembled the typhoid germ in culture characters or in agglutinating tests.

TABLE XXXIV.

RESULTS OBTAINED FROM CULTURES MADE FROM PARCHMENT SACS AFTER VARYING PERIODS OF EXPOSURE IN RIVER WATER.
SERIES INFECTED WITH "Y" CULTURE.

No. of Sacs	Approximate Dosage of Typh. Bacilli per c.c.	Time after Inoculation When Examined—Days	Total No. of Colonies on Plates Made	No. of Colonies Fished	No. of Typhoid Colonies
6.....	1,000	1	19	8	3
		3	25	6	2
		14	68	9	0
		17	6		
7.....	2,000	4	3,600	9	0
		12	28	5	0
		14	185	5	0
		17	15	6	0
8.....	4,000	6	8	0
		10	73	6	0
		12	35	4	0
		17	47	8	0
9.....	10,000	4	11,500	11	0
		7	43	7	0
		10	160	10	0
		12	107	5	0
10.....	20,000	1	158	8	4
		3	100	16	10
		5	18	0
		7	103	6	0
		10	150	7	0

The "y" series was placed in the river at the same time as "x." Practically the same results were secured. In all the sacs examined up to the third day after immersion, the typhoid organism was detected where both light and heavy seeding was used. In none of the examinations after the third day (sixteen in number) was any evidence found which would lead to the belief that this specific germ had retained its vitality in any of the sacs for a longer period than three days.

TABLE XXXV.

RESULTS OBTAINED FROM CULTURES MADE FROM PARCHMENT SACS AFTER VARYING PERIODS OF EXPOSURE IN RIVER WATER.

SERIES INFECTED WITH "z" CULTURE.

No. of Sacs	Dosage of Typh. Bacilli per c.c.	Time after Inoculation When Examined—Days	Total No. of Colonies on Plates Made	No. of Colonies Fished	No. of Typhoid Colonies
12.....	540	1 24	50	3	3
		1	360	17	15
		2	350	12	6
		5	100	8	0
		12	4	2	0
13.....	540	7	7	3	0
		9	4	0
		12	7	0
14.....	5,400	1 24	53	3	3
		7	6	1	0
15.....	10,000	1	1,500	15	14
		2	4,000	13	6
		5	750	11	0
		10	430	12	1
		12	3	2	0

In this third series, in which the "z" culture isolated from feces was used, the seedings were in each case only about one-half of what was employed in the other tests. This test was not continued for as long a period as the other two, but the specific organism was recovered easily from all tubes examined shortly after inoculation, also on the first and on the second day. No tests happened to be made between the second and the fifth day, but on the tests made on this latter date and subsequently the typhoid organism was not found in any case, with one single exception. On the ninth day, among twelve colonies removed from sac No. 15, containing the maximum amount of typhoid seeding, one was found which responded to all the cultural tests for typhoid as well as the agglutination test in dilutions of 1:1,000. Examinations made from this same sac on the twelfth day showed no similar colonies. It is impossible to explain the presence of this single colony, appearing by itself eight or nine days later than any other typhoid colonies in this or any other sac. It is well known, of course, that the vitality of all individual organisms of the same species is not absolutely the same, but it seems highly improbable that the appearance of this single germ,

among the hundreds of colonies examined subsequent to the third day, can be attributed to a protracted retention of vitality much beyond the usual period.

A general summary of the foregoing detailed results, as presented in the appended tables, may perhaps aid in the interpretation of the analyses, if they are grouped according to the time of exposure. In Table XXXVI is shown the typhoid findings in the different sacs on the different days in which they were examined.

TABLE XXXVI.

NUMBER OF TYPHOID ORGANISMS RECOVERED FROM DIFFERENT SACS ON DIFFERENT DAYS.

Series	Sac No.	1D	2D	3D	4D	5D	6D	7D	8D	9D	10D	11D	12D	13D	14D	15D	16D	17D
X	1.....	1	..	0	..	0	0
	2.....	0
	3.....	0	0
	4.....	..	2	..	0	0	0
Y	5.....	0	2	0	0	0	0	0	0	0
	6.....	3	2	0
	7.....	..	0	0	0	0	0	..	0
	8.....	0	0	..	0	0	..
Z	9.....	0	0	0	0
	10.....	4	..	10	..	0	..	0	0
	12.....	31 15	6	0	0
	13.....	0	..	0	..	0
	14.....	31	0
	15.....	14	6	0	1	..	0

Table XXXVII gives the total number of colonies from all sacs that were tested from day to day with the typhoid findings; also the distribution of the typhoid findings among the respective sacs.

TABLE XXXVII.

GENERAL SUMMARY OF RESULTS OBTAINED FROM EXAMINATIONS OF TYPHOID-INFECTED SACS MADE ON SUCCESSIVE DAYS.

	1	2	3	4	5	6	7	9	10	13	14	16	17	Totals
Number colonies tested by sub-cultures from all sacs.....	72	25	44	41	61	28	32	18	46	38	25	14	15	459
Number typhoid colonies found in various sacs on successive days.....	43	12	14	0	0	0	0	1	0	0	0	0	0	70
Total number times all sacs were examined.....	8	2	5	4	6	3	6	2	5	7	3	2	1	54
Total number times in which typhoid colonies were found..	7	2	3	0	0	0	0	1	0	0	0	0	0	13

¹ Indicates cases where examination was made one hour after examination.

From the foregoing tables it appears that there is a striking uniformity in the results which have been obtained in all three series made with different strains of the typhoid organism derived from entirely different sources. In each case the inoculated organism persisted in the sacs containing the minimum and maximum amount of seeding for the same period of time.

Vitality was retained as a rule in the waters of the Illinois river no longer than an interval of three days after infection. In only a single instance was any typhoid germ isolated after this period, and that case was on the ninth day. A rational explanation for the presence of this single colony is not apparent, but, in the judgment of the writer (H. L. R.) the presence of this particular organism is not believed to have any special significance in the problem under consideration.

GENERAL CONCLUSIONS.

1. From the experiments recorded in this paper it appears that under conditions that probably closely simulate those in nature the vast majority of typhoid bacilli introduced into the several waters studied perished within three to four days.

2. It is theoretically possible that specially resistant cells may occur which are able to withstand for a longer period the hostile influences evidently present in water. Our experiments, however, show that if such resistant individuals exist they must be very few in number and constitute only a small fraction of the bacilli originally entering the water.

3. It is not the intention of the writers to claim that the behavior of typhoid bacilli under the conditions herein described is representative of all conditions obtaining in all natural bodies of water.

THE QUESTION OF VIRULENCE AMONG THE SO-CALLED PSEUDODIPHTHERIA BACILLI.*

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THE "pseudodiphtheria bacillus," since the year 1887 when Loeffler and Hoffmann-Wellenhof isolated it, has been the subject of much controversy, and even now it is a matter of dispute whether the organism thus designated is simply an attenuated *B. diphtheriæ* or, as Loeffler thought it, a member of the same group as the latter, but of a separate species. Behring, Ritter, Roux, and Yersin belong to the school which holds that there is no essential difference between the virulent and non-virulent organisms, and recently Westbrook in this country has appeared as a strong adherent of the same theory. On the other hand, the majority of bacteriologists, among them Loeffler, Hoffmann-Wellenhof, Escherich, Zarniko, Fraenkel, Spronck, Kruse, Flügge, and Beck, hold that *B. diphtheriæ* belongs to a large group of organisms consisting of several distinct species. On the whole, in spite of Behring's recent utterances, the trend of opinion is in the latter direction.

The earlier investigators thought it a comparatively easy matter to distinguish the pseudodiphtheria bacillus from *B. diphtheriæ*, for the former was said to have certain cultural and morphological characteristics quite different from the latter, and also to differ from it in chemical activity and in virulence for animals. Of late, however, each one of these distinguishing characteristics has been called in question, and the distinction between the two organisms, far from growing plainer, becomes less and less assured, until it seems questionable whether there is any one point which can always be relied upon to distinguish them.

Loeffler† and Hoffmann-Wellenhof thought that the appear-

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† It seems unnecessary to publish a bibliography of this subject, in view of the fact that there are already several fairly exhaustive ones appended to recent publications. The reader is referred to SCHABAD, *Jahrb. f. Kinderheilk.*, 1901, 54, p. 381; LEWANDOWSKY, *Centrabl. f. Bakt.*, 1904, 36, p. 339; and, for English articles, to GRAHAM-SMITH, *Jour. of Hyg.*, 1904, 4, p. 258.

ance of the growth upon solid media was enough to distinguish the pseudodiphtheria bacillus, but we find that, according to the description given by Loeffler and also by Fraenkel, Barber, and Sudeck, the growth on agar is scanty, grayish, translucent, while Hoffmann-Wellenhof and the greater number of investigators describe it as abundant, white, moist, and shining. Moreover, it is now generally recognized that *B. diphtheriæ* itself may vary so greatly in its cultural characteristics as to be indistinguishable in this way alone from the allied bacteria. In a recent article Lewandowsky has endeavored to simplify matters by emphasizing the typical cultural differences between the two forms, but a careful comparison of the results obtained by different observers will convince any reader that variations from the usual type are so frequent as to render certainty on this ground alone impossible.

Hoffmann described the pseudodiphtheria bacillus as shorter and thicker than the bacillus of true diphtheria, and this statement is concurred in by Escherich and by most observers. Schabad insists that the morphology of the pseudodiphtheria bacillus from a twenty-four-hour serum culture is one of its most distinguishing characteristics. According to Schabad's description, the bacillus in question is very different from the Klebs-Loeffler bacillus, not only shorter and thicker, but devoid of granules and bars, and arranged in parallel rows, in contrast to the radiating arrangement of *B. diphtheriæ*. On the other hand, Roux and Yersin, Kruse, Fraenkel, Schanz, and Westbrook insist that it is impossible to distinguish the two by their appearance under the microscope, and Januszewska and Beck give the parallel arrangement of the rods as typical of the true, not the pseudo bacillus. Westbrook, who has published the results of the study of 608 cases of clinical diphtheria, found that the short, solid bacilli, devoid of granules or bars, corresponding to the description usually given of the pseudodiphtheria bacillus, was often found in the later stages of diphtheria, sometimes in the earlier stages, and, in a few cases, was the only form present throughout the disease. An interesting view of the subject is given by A. P. Ohlmacher in his article "On Some Experimentally Produced Morphologic Variations in *B. diphtheriæ*." Ohlmacher holds that the pseudodiphtheria bacillus is only a modified variety of *B. diphtheriæ*, and that this modification may be brought about by a sojourn in the body of a relatively immune animal. A strain which originally consisted of barred and granular forms, changed to the short, solid type after passage through a white rat, but assumed its original character again after the passage through the highly susceptible guinea pig. Conversely, a strain originally composed of the short, solid, plump rods was changed to a typical *B. diphtheriæ*, with greatly heightened virulence, by passage through a guinea pig.

The question as to the value of Neisser's stain is also unsettled. While Schabad, Franke, Auckenthaler, and Beck consider this a very valuable aid

to diagnosis, others declare it most unsatisfactory. Bütschli demonstrated Neisser granules in several kinds of bacteria. Kurth isolated three strains of indubitable, virulent diphtheria bacilli which had no Neisser granules. Schwoner, Spronck, and Graham-Smith think the method is of little value, and Westbrook has abandoned it in favor of Loeffler's methylene blue.

The fact, first observed by Zarniko, that the diphtheria bacillus causes an active production of acid in broth cultures, while the pseudodiphtheria bacillus causes either a faintly acid reaction followed quickly by an alkaline, or an alkaline reaction from the outset, has been regarded by some as an important, if not the most important, difference between the two organisms. Escherich has even made the statement that in the presence of an alkaline reaction it is unnecessary to test the pathogenicity of a culture for guinea pigs, for it is an absolute rule that the acid-producers are virulent and the non-acid-producers avirulent — a statement in which Graham-Smith concurs.

Exceptions to this rule, however, are numerous enough to invalidate it as an absolute test. Neisser, who agreed with Escherich as to all diphtheria bacilli forming acid, found an acid-producing, pseudodiphtheria bacillus. Spronck found that not all diphtheria bacilli form acid; Roux and Yersin, that both kinds form first acid and then alkali, the pseudodiphtheria bacillus forming alkali more rapidly than the true diphtheria. Slawyck and Manica-tide came to the same conclusion. Kurth, Peters, Lehmann and Neumann found pseudodiphtheria bacilli which formed as much acid as the true *B. diphtheriæ*. Schabad and Neisser, however, think that by careful titration the two can always be distinguished.

Loeffler considered virulence for guinea pigs the most unvarying characteristic of the organism which has received his name, and Escherich concurred in this. Edema of the subcutaneous tissue, infiltration or hemorrhage at the point of inoculation, pleuritic effusion, hyperemia and enlargement of the adrenals, hyperemia of liver and kidneys, without any change in the spleen, are the typical lesions produced by the injection under the skin of a few drops to one cubic centimeter of a broth culture of the bacillus diphtheriæ. Death occurs usually in one to three days. By many bacteriologists all virulent diphtheria-like organisms are considered true diphtheria bacilli; all non-virulent, pseudodiphtheria. That the question is not as simple as this, however, was shown by Roux and Yersin, who succeeded in artificially lowering the virulence of diphtheria bacilli; and by Brieger, Fraenkel, Lubowski, Schabad, and Westbrook, who showed that typical bacilli from cases of virulent diphtheria sometimes prove non-virulent to guinea pigs. Nor is it true that the pseudodiphtheria bacillus is always non-virulent. Spronck, Fraenkel, Zupnik, Gelpke, and others find that a slight degree of virulence for guinea pigs is a property of many strains of the pseudodiphtheria bacillus, and that these strains cannot be distinguished by ordinary means from feebly virulent strains of the true diphtheria bacillus. Some observers even claim a pathogenicity for man in certain cases. Goldscheider isolated pseudodiphtheria bacilli from five cases of angina, with fever and swollen lymphatic glands, and thought these organisms the cause of the symptoms. Warnecke found similar bacilli in pure culture in three cases of otitis media, and in one of these cases he found the same organism in the exudate of meningitis and

in metastatic abscesses in the lung. Kruse and Pasquale cultivated a pseudodiphtheria bacillus (*B. clavatus*) from the organs of several cases of Egyptian dysentery, and Sanfelice found one in the pustules of smallpox. In all these cases the organisms were non-virulent to guinea pigs.

Soon after the introduction of the agglutination test for the diagnosis of typhoid fever efforts were made to apply the method to the clinical diagnosis of diphtheria, but so far only one experimenter has done so with marked success. Schwoner succeeded in immunizing a horse in such a way as to produce a serum which agglutinated different strains of diphtheria bacilli in dilutions as high as 1:10,000, but not the pseudodiphtheria bacillus. Lubowski, by using non-virulent diphtheria bacilli, obtained a serum which agglutinated twenty-three other strains, but irregularly and not in dilutions over 1:160. Nicholas, Bruno, and Lesieur, after many experiments, declared the agglutination test of no value.

Finally Martini tried to distinguish the true from the pseudo bacilli by the bactericidal action of diphtheria antitoxin on the diphtheria bacillus. He stated that the latter would not grow in such serum, while the pseudodiphtheria bacillus would: but this has since been disproved. Fraenkel, Spronck, and Landsteiner have shown that the antitoxic serum is not bactericidal to diphtheria bacilli, and that there is no difference between the two organisms in this respect. Graham-Smith endeavors to simplify the question by making three classes of organisms: first, *B. diphtheriæ*, which is always pathogenic to guinea pigs and always produces acid in sugar broth; second, the pseudodiphtheria bacillus, never pathogenic and never producing acid; and, third, a new group, *B. diphtheroides*. It is not quite clear how Graham-Smith distinguishes this last from the pseudodiphtheria bacillus. Certainly the strains described by him differ not at all from many so-called pseudodiphtheria bacilli in the literature.

Therefore, according to our present state of knowledge, we must either consider the Klebs-Loeffler bacillus, and those resembling it, as essentially the same organism, and their individual peculiarities as unimportant; or, if this seems not in accordance with our observations and we insist upon recognizing different varieties, we must admit that every method of differentiating *B. diphtheriæ* from the closely allied forms has failed in the hands of one observer or another, and that we are still waiting for one which will be absolutely decisive.

According to Beck, and to the latest utterances of Loeffler and Spronck, it is impossible to distinguish the true from the pseudo bacillus by means of cultural peculiarities morphology, presence of Neisser granules, chemical activity, or virulence for animals; and the only hope for a sure method of differentiation lies in the application of specific sera to neutralize specific toxins. We know that in clinical experience the reaction to the diphtheria antitoxin is often a very valuable aid to diagnosis, for the failure of such a reaction shows that the symptoms in the case were not caused by the specific toxins of the diphtheria bacillus. Experiments to demonstrate the fact that the pseudodiphtheria bacillus does not form these toxins were made by Glückmann and by Neisser, who showed that animals treated by repeated injections of pseudodiphtheria bacilli were not thereby rendered immune to *B. diphtheriæ*.

The most important and suggestive work in this direction has been done by Spronck. He proved that the xerosis and the pseudodiphtheria bacillus are not attenuated forms of *B. diphtheriæ*, because they do not form the same toxins as the latter. He injected guinea pigs with cultures of xerosis and pseudodiphtheria bacilli which were feebly virulent, causing local edema, loss of weight, loss of appetite, etc.; and the same symptoms appeared in the animals which received a dose of Behring's antitoxin sufficient to protect against a lethal dose of the diphtheria bacillus, as in those which received no antitoxin, showing that the toxins formed by the xerosis and pseudodiphtheria bacilli are not neutralized by the diphtheria antitoxin. This test, which seems both logical and conclusive, is rejected by Schabad, who still insists that differentiation between the two organisms must rest upon morphological and chemical properties, upon the difference in shape, in Neisser granules, and in acid productions in broth cultures. Spirig also rejects Spronck's method, but on questionable grounds. Having isolated from the throat of a normal child a bacillus, resembling in appearance and growth the pseudodiphtheria bacillus, he tested it by Spronck's method, and found that, as in the case of Spronck's organisms, the Behring antitoxin did not protect against the effects produced by the bacillus. Nevertheless, Spirig continued to regard the organism as a feebly virulent *B. diphtheriæ*, chiefly on the ground that it was found in the throat of a child exposed to diphtheria in the course of a rather extensive house epidemic of this disease.

The following is an account of the work done in the Memorial Institute for Infectious Diseases, first by Dr. E. H. Ruediger and later by myself, in the course of which it has appeared probable that the method suggested by Spronck is the most satisfactory one for distinguishing *B. diphtheriæ* from the pseudodiphtheria bacillus, and that by this method it is possible also to prove that the term "pseudodiphtheria" covers not only one bacillus, but a group of bacilli.

During the months from March to July, 1903, Dr. E. H. Ruediger succeeded in isolating from the throats of seven fatal cases of scarlatina, which came under his observation in the Memorial Institute for Infectious Diseases, organisms which resembled *B. diphtheriæ*, yet differed from it in several important respects. I quote from Dr. Ruediger's description as follows:*

"Clinically these cases were variously diagnosed as diphtheria, gangrenous tonsillitis, and pharyngitis complicating scarlatina, black scarlet fever, and black diphtheria. The bacteriologic examination in each case made by Dr. Weaver showed "pseudodiphtheria bacilli." Five cases were treated with antidiphtheria serum, but the effects were always bad. All symptoms were greatly aggravated, an experience to which Johannessen called attention

* *Trans. Chicago Path. Soc.*, 1903, 6, 45.

years ago. In two of the cases, Nos. 1 and 7, the effects of the antidiphtheria serum were so marked that it was looked upon as having materially shortened life. Case 7 died within eighteen hours after receiving 10 c.c. of serum valued at 4,000 units. The two cases which did not receive the antidiphtheria treatment died within a few hours after admission to the hospital.

From the throats of all these cases was isolated a bacillus which resembles the true diphtheria bacillus, but shows some cultural differences. This organism was found in the throats in great abundance. The seven strains cannot be distinguished from one another either culturally or morphologically. They differ from true diphtheria bacilli and from non-pathogenic pseudodiphtheria bacilli in the following particulars:

Diphtheria Bacillus	Organism No. 5—Pathogenic Pseudodiphtheria Bacillus	Non-pathogenic Pseudodiphtheria Bacillus
BROTH Not turbid; white scum on surface; sediment on bottom; clumps adherent to sides of tube.	BROTH Uniformly turbid.	BROTH Uniformly turbid.
AGAR Rather dry, grayish growth.	AGAR Soft, moist, and whitish growth.	AGAR Opaque whitish growth.
POTATO No visible growth.	POTATO A hardy, light-brown growth.	POTATO Dirty, grayish, glistening growth.
LITMUS MILK No change.	LITMUS MILK Milk is turned white in five to six days.	LITMUS MILK No change.

These organisms are agglutinated by the serum of a rabbit which had received three injections of a twenty-four-hour bouillon culture of organism No. 5. This strain is agglutinated in dilutions up to 1:500, while the others are clumped only in dilutions up to 1:200. The non-pathogenic pseudodiphtheria bacillus is not agglutinated by this serum. All agglutination tests were macroscopic. Guinea pigs are not protected against these organisms by antidiphtheria serum. Neisser's stain is of no value in differentiating them from true diphtheria bacilli. All seven strains are pathogenic for guinea pigs, after having been kept on agar for several months, when injected intraperitoneally in doses of 4 to 5 c.c. The virulence of organism No. 5 was increased by passage through guinea pigs. After having been passed through four animals, 1.5 c.c. of a twenty-four-hour bouillon culture killed a 250 g. guinea pig within twelve hours.

The post-mortem findings in the guinea pigs may be summed up as follows: The serous cavities contain a moderate quantity of fluid. The liver, spleen, and kidneys are markedly congested. The organism can be isolated from the peritoneal cavity, the heart's blood, and the internal organs.

Two guinea pigs, (a) and (b), weighing 650 g. each, were now inoculated with the virulent organism No. 5. Each animal received 2 c.c. of a twenty-four-hour bouillon culture, intraperitoneally, and (b) got at the same time 2 c.c. of antidiphtheria serum, valued at 250 units per cubic centimeter. Guinea pig (b) was found dead in eighteen hours after inoculation, while (a)

was apparently healthy on the second day. Guinea pig (*a*) was now given 2 c.c. of antidiphtheria serum and died within twelve hours. This antidiphtheria serum protected guinea pigs against three or four times the minimum fatal dose of true diphtheria bacillus, control animals dying in twelve to twenty-four hours.

I now undertook to prepare an antitoxic serum for this organism. A large rabbit was injected at intervals of seven days with 1, 2, and 3 c.c. of a filtered bouillon culture of organism No. 5. A week after the third injection several cubic centimeters of blood were withdrawn from this rabbit, and also from a normal rabbit, allowed to clot, and the sera collected under aseptic precautions. Eight guinea pigs were now injected intraperitoneally with 3 c.c. of a twenty-four-hour bouillon culture of organism No. 5. Immediately after the inoculation, two of these animals were given 2 c.c. of antidiphtheria serum valued at 250 units per cubic centimeter. Two were given 2 c.c. of normal rabbit's serum, and two others 2 c.c. of the immune rabbit's serum. The remaining two animals received no treatment whatever after the inoculation. All of these animals died within twenty four hours, except those two which had been treated with the immune rabbit's serum. These two recovered."

A short abstract of this work of Dr. Ruediger's is given in the last article by Graham Smith, who dismisses it with the remark that "the author . . . appears to use the term 'pseudodiphtheria' bacillus as equivalent to the non-virulent diphtheria bacillus." As the bacillus in question was shown to be decidedly virulent, and was proved to be not a strain of the *Bacillus diphtheriae*—inasmuch as the diphtheria antitoxin failed to protect against it—it is difficult to see why Graham Smith would have it designated as "a non-virulent diphtheria bacillus."

Dr. Ruediger's work was broken off at this point by his departure for Manila, and at Dr. Hektoen's request I took it up, with a view to discover how often this organism could be found in cases of diphtheroid pharyngitis in scarlet fever. It so happened, however, that the scarlet fever in Chicago this year was of an 'exceedingly mild type, and but two cases of pseudomembranous scarlatinal pharyngitis came under observation at the Memorial Institute, both of which proved to be due to virulent streptococcal infection. For lack of this material, I undertook a routine examination of the throats of all the scarlatina patients who had angina of more than usual severity both in this hospital and the Cook County Hospital, making in all thirty-two cases between January 1 and June 1, 1904. From twelve of these, organisms resembling more or less *B. diphtheriae* were isolated and studied. Eighteen cases of diphtheria, all but two of them from the Cook County Hospital, were also examined, and eight of

these proved atypical in one of two ways; either the organism isolated did not correspond to the usual description of *B. diphtheriæ*, or the clinical history showed that the administration of diphtheria antitoxin had not been followed by favorable results. Four cases diagnosed as diphtheria complicating scarlet fever were examined and pseudodiphtheria bacilli isolated from two; four of diphtheria complicating measles, with pseudodiphtheria bacilli in three; one case diagnosed as scarlet fever and measles combined, and three cases of measles with unusually severe pharyngitis, all of which yielded pseudodiphtheria bacilli. Finally from one normal throat and from one case of tonsillitis, occurring in the course of acute inflammatory rheumatism, bacilli of the same kind were isolated.

The number of cases of scarlet fever in which the so-called pseudodiphtheria bacillus was found is small—only eleven out of thirty-one cases—but I do not think that this represents the real proportion of positive cases. It is probable that in the earlier part of the work a faulty technic was responsible for the failure to find pseudodiphtheria bacilli in a larger number of cases. The first cultures were made on Loeffler's blood serum, upon which medium the pseudodiphtheria bacillus usually grows more slowly than the diphtheria bacillus, but when once started grows more abundantly than the latter. It happened several times that serum cultures twenty-four to forty-eight hours old, from a throat the smears from which had shown the presence of suspicious bacilli, would fail to show anything but cocci, and the cultures would then be rejected. Happening once to re-examine such a culture after seven days, I found it full of slender curved and straight rods with bipolar granules. After that, the first cultures were made on glycerin agar as well as upon serum, and, in case bacilli were not found in either, a loopful of the serum culture was transferred to a tube of broth, and from this tube a loopful to the water of condensation at the bottom of a tube of glycerin agar, and the tube tipped to allow the water to run over the inclined surface of the agar. From such a culture colonies of bacilli could usually be obtained, and, in case of failure, the original serum culture was allowed to stand at room temperature for four or five days, and the procedure repeated. By this means it was found possible

DIAGRAM NO. 1.

No.	Diagnosis	Morphology, 18 Hrs. Serum	Nettleser's	Morphology, 1-8 Days Serum	Loeffler's Blood Serum	Glycerin Agar Slant	Potato	Broth, 24 Hrs.	Virulence for Guinea Pigs	Remarks
1.....	Tonsillitis; acute articular rheumatism.	Short, solid, wedge-shaped rods.	-	Same as in 18 hrs.	Abundant, buff.	Abundant, white.	Scanty, white.	Cloudy, alkaline, later acid.	Pure culture from tonsils.
2.....	Measles and diphtheria.	Short and medium, solid and barred rods.	-	Do.	Grayish colonies.	Abundant, white.	No growth.	Cloudy, faintly acid.	Slight loss of weight.
3.....	Do.	Do.	-	Do.	Slow, white, later orange.	Abundant, white, then orange.	Slow, scanty, orange.	Cloudy, alkaline.	Slight loss of weight.	Nos. 2 and 3 were from the throats of two sisters; no membrane.
4.....	Scarlet fever.	Slender, solid, and long granular rods.	-	Typical diphtheria bacilli.	Abundant, colorless.	Abundant, colorless.	Abundant, colorless.	Cloudy, neutral.	Obtained by aspiration from swollen tonsil.
5.....	Do.	Short, solid, wedge-shaped rods.	-	Same as in 18 hrs.	Grayish colonies.	Abundant, white.	No growth.	Cloudy, acid.
6.....	Do.	Short, thick rods; bipolar granules.	-	Do.	Grayish colonies.	Scanty, colorless.	No growth.	Cloudy, alkaline.	Severe angina; many streptococci.
7.....	Do.	Short, wedge-shaped, and long, slender rods.	-	Do.	Grayish colonies.	Scanty, colorless.	No growth.	Cloudy, alkaline.
8.....	Measles.	Short, slender, pointed rods.	-	Do.	Moist, colorless smear.	Scanty, colorless.	No growth.	Cloudy, alkaline.	Severe angina; many streptococci.

9.	Scarlet fever ?	Short and long, barred and solid, rods.	-	Typical diphtheria bacilli.	<i>Grayish colonies.</i>	<i>Scanty, colorless.</i>	<i>No growth.</i>	<i>Clear, acid.</i>	Tonsilitis; rash atypical.
10.	Diphtheria.	Short, wedge-shaped, and swollen rods.	-	Short, granular, and solid; long, barred.	Abundant, creamy, later yellow.	Abundant, yellow.	Abundant, yellow.	Cloudy, acid.	Slight loss of weight.	Antitoxin had no effect in this case.
11.	Do.	Long, slender, clubbed and pointed, solid rods.	-	Typical diphtheria bacilli.	<i>Moist, colorless smear.</i>	<i>Scanty, colorless.</i>	Slow, scanty, white.	Cloudy, acid.	Bacillus isolated after membrane had disappeared.
12.	Scarlet fever.	Short, granular, and solid rods.	+	Typical diphtheria bacilli.	<i>Grayish colonies.</i>	Abundant, white.	<i>No growth.</i>	Cloudy, faintly acid.
13.	Do.	<i>Typical diphtheria bacilli.</i>	+	Typical diphtheria bacilli.	Abundant, creamy, liquefying.	Abundant, colorless.	Abundant, yellowish.	<i>Clear, acid.</i>	Obtained in pure culture from an oral discharge in 3d week.
14.	Do.	<i>Typical diphtheria bacilli.</i>	+	Typical diphtheria bacilli.	<i>Grayish colonies.</i>	<i>Grayish colonies.</i>	<i>No growth.</i>	<i>Clear, acid.</i>	Slight loss of weight.	Severe angina; no membrane.
15.	Diphtheria.	Slender, barred, and solid rods.	-	Typical diphtheria bacilli.	<i>Moist, colorless smear.</i>	Abundant, gray; agar turns dark brown.	Abundant, gray, shining.	Cloudy, alkaline, later acid.	Slight loss of weight.	From same throat as No. 1 of 2d series.
16.	Scarlet fever.	Short, slender, solid rods; few small granules.	-	Short, slender rods, granules.	Abundant, liquefying.	Abundant, green; agar turns purple.	Dry, dark brown.	Cloudy, neutral, later acid.
17.	Do.	Short, solid, long barred rods.	-	Same as 18 hrs.	Abundant, liquefying.	Abundant, green; agar turns purple.	Shining, reddish, abundant.	Cloudy, acid.

to isolate pseudodiphtheria bacilli in all cases in which the original smears had shown their presence. Schabad has called attention to this slowly developing and finally abundant growth on serum of the pseudodiphtheria bacillus. Another source of error is the fact, pointed out by Kurth, that these bacilli, when grown in mixed culture with cocci and other varieties, form much shorter rods than in pure culture. Indeed, they may sometimes look like lance-shaped cocci in the mixed cultures.

At the outset in such an examination one encounters the question as to what is to be considered a pseudodiphtheria bacillus. Usually this term is applied to bacilli which resemble more or less *B. diphtheriæ*, but are shorter and thicker than the commoner types of the latter. Many describe them as short, wedge-shaped rods, often lying in pairs with their bases facing each other (Wesbrook's Type D 2). Probably this is the same form as the one described by Graham-Smith: "darkly stained, oval, with one narrow, unstained septum." By others the typical pseudodiphtheria bacillus is said to be short and thick, and to contain granules whose diameter is smaller than that of the bacillus. There are, however, authors who give this name to bacilli which do not in the least resemble the bacillus of diphtheria and could never be mistaken for it—large, thick rods, devoid of bars or granules, either staining solidly or with a clear spot in the center. Such is the pseudodiphtheria bacillus of Spirig and of Schwoner, and such a bacillus is shown in one of the cuts in Schabad's article. These large rods with clear center were encountered very frequently in the throat cultures examined by me, but they were ignored, for there seemed no grounds for considering them pseudodiphtheria bacilli, as they do not resemble either morphologically or culturally the Klebs-Loeffler bacillus. Only those organisms were isolated which corresponded to one or the other of the types described and pictured by Wesbrook, namely, granular or barred rods, of varying length or outline, and solid rods, slender, curved or straight, spindle-shaped, clubbed, or wedge-shaped.

Thirty-one such bacilli were isolated, fourteen of which proved pathogenic to guinea pigs, seventeen non-pathogenic. Diagram

1 gives a tabulation of the seventeen non-pathogenic organisms. For brevity's sake, the term "typical diphtheria bacillus" is used to describe a polymorphous bacillus containing granules staining red by Loeffler's methylene blue and presenting the usual variety of forms, including spindles, clubs, and jointed rods. Deviations from the typical pseudodiphtheria bacillus are entered in italics.

Of all these seventeen organisms only two, Nos. 13 and 14, were diagnosed as diphtheria bacilli from the eighteen-hour serum culture. No. 13 was from the aural discharge of a scarlet fever patient, and was found in pure culture. It differed so much in its cultural characteristics from the Loeffler bacillus, liquefying both gelatin and blood serum, that it could easily be distinguished from the latter.

No. 14 corresponded in morphology and culture with *B. diphtheriæ*, but the clinical history did not point to diphtheria. There was no membrane in the throat, and none of the children in the same ward developed diphtheria. Two successive doses of this organism—the first as large as 1 per cent. of the body weight, the second 2 per cent.—caused no symptoms in guinea pigs, only a slight loss of weight. It is, of course, possible that this was an attenuated *B. diphtheriæ*.

No. 12 was hardly typical enough to be pronounced an undoubted *B. diphtheriæ*, especially as clinical confirmation was wanting. The short, solid forms predominated, nevertheless Neisser granules were present, and in its growth on all media except agar it corresponded to *B. diphtheriæ*.

The other fourteen bacilli represented the forms which we are accustomed to regard as typical of *B. pseudodiphtheriæ*, although, as we have already seen, Westbrook claims the solid wedges and slender rods as among the commonest types of the true diphtheria bacillus.

There is a great variety in the growth of these different organisms on solid media, some growing abundantly, like the typical pseudodiphtheria bacillus, others more like *B. diphtheriæ*. Three of them, Nos. 13, 16, and 17, liquefied serum and gelatin, as did Klein's "*B. diphtheroides*," and Graham Smith's "*B. diphtheroides liquefaciens*." Nos. 15, 16, and 17 differed from all the

others in turning agar a deep brownish or purplish color—a property which both Prochaska and Escherich considered characteristic of the pseudodiphtheria bacillus.

Neisser granules were present in Nos. 12, 13, and 14 only. Seven of the seventeen developed in old serum culture typical diphtheria-like forms—a fact which Schabad has also noted of the organisms studied by him.

All were entirely non-pathogenic for guinea pigs, except Nos. 2, 3, 10, 14, and 15, which, in doses of 1.5 to 2 per cent. of the body weight, caused a transient loss of weight. Apparently Nos. 1, 4, 9, and 13 were pathogenic to man. No. 1 was found in pure culture on the tonsils of a case of acute articular rheumatism with tonsillitis. A similar organism was found by Zarniko in a similar case. This pseudodiphtheria bacillus was typical in morphology and culture, and in absence of virulence for guinea pigs, yet it was apparently the cause of a severe tonsillitis in a woman.

No. 4 was obtained by aspiration of a swollen tonsil which was supposed to be the seat of suppuration. The few clear drops thus drawn contained a pure culture of a bacillus resembling in many ways *B. diphtheriæ*, but differing from it in the absence of Neisser's granules, in growth on potato, and in absence of virulence for guinea pigs.

No. 9 was found in large numbers, mixed with a few cocci, in the throat of a child who was suffering from a severe tonsillitis with a slight red rash over the body. A positive diagnosis was never made in this case. The child had a history of several such attacks, but she did not contract scarlet fever after several weeks' sojourn in the scarlet-fever ward.

Finally, No. 13 was obtained in pure culture from the aural discharge of a convalescent scarlet-fever patient. Seen in stained specimens it was apparently a typical *B. diphtheriæ*, but the abundant, moist growth, the liquefaction of serum, and the absence of virulence for guinea pigs served to distinguish it from the latter.

Turning now to the sixteen pathogenic organisms, a word of explanation is needed as to the method employed in determining virulence. Dr. Ruediger had found that his strains of pseudodiphtheria bacillus killed guinea pigs of 500–600 g. in doses of 5

c.c. of broth culture injected into the peritoneum. One organism, No. 5, he succeeded in rendering more virulent by successive passages through guinea pigs, but when it reached my hands this increased virulence had already disappeared, and it required 4 c.c. to kill a 500 g. guinea pig. Following his example, I used intraperitoneal injections instead of subcutaneous, beginning with a dose equal to 1 per cent. of the body weight, and increasing or diminishing it according to the result. Post-mortem examination of the animals showed that the organisms produced two different effects, one of which corresponds more to that caused by the intraperitoneal injection of diphtheria bacilli, the other to that caused by the injection of Ruediger's bacillus. The first class caused death in forty-eight hours, rarely sooner, sometimes after four to eight days, in which case the animals became emaciated, and in one case (No. 6) showed typical post-diphtheritic paralysis. Animals living more than three days usually yielded no cultures at all; those dying more quickly yielded cultures from the site of inoculation, from the peritoneum, and rarely from the blood or organs. The changes found in these animals were usually infiltration or hemorrhage at the site of inoculation, rarely pleuritic effusion, but usually a clear, reddish, or gelatinous exudate in the peritoneum. The upper small intestine was collapsed and red, the liver and kidneys somewhat congested, the spleen unchanged, and the adrenals large and dark red.

Animals injected with Ruediger's bacillus, and organisms resembling it, died almost always within eighteen to twenty-four hours. The body was extraordinarily limp, and there was seldom any infiltration at the site of inoculation. The peritoneum contained a large quantity of clear, reddish fluid, the liver and kidneys were much congested, the latter often dripping blood on section. The spleen was unchanged, and there was no change in the adrenals. The bacilli could be isolated from the peritoneal and pleural fluids, the blood, urine, and all the organs, and from the subcutaneous tissue in a few cases. This general bacteriemia occurred also when the injection was made into the subcutaneous tissue. According, therefore, to the effect on guinea pigs, we have here two distinct classes of organisms.

DIAGRAM NO. 2.

No.	Diagnosis	Morphology, 18 Hrs. Serum	Neisser's Granules	Morphology, 4-8 Days Serum	Growth on Solid Media	Broth, 24 Hrs.	Virulence for Guinea Pigs	Bacillus, Recovered	Action of Anti- toxin	Action of Ruediger Serum	Remarks
Dr. Ruediger's No. 1 st	Scarlet fever.	Regular, short, and long granu- lar.	+	Same as 18 hrs.	Abundant on all.	Cloudy, alkaline.	Less than 1% killed in 24- 48 hrs.	From all or- gans and fluids.	-	+
1.....	Diph- theria.	Short, slender, solid rods.	-	Typical diph- theria bac- illi.	Like diph- theria.	Cloudy, acid.	1% killed in 48 hrs.	From peri- tonium; few colonies from blood.	+	-
2.....	Measles and scar- let fever.	Short and long, slender, solid rods.	-	Same as 18 hrs., with short granu- lar and long- barred.	Abundant, creamy on serum and potato.	Cloudy, acid.	1% killed in 4 days; 2% in 24 hrs.	From all or- gans and fluids; typ- ical diphthe- ria bacilli.	+	-
3.....	Diph- theria.	Short, solid rods.	-	Typical diph- theria bac- illi.	Like diph- theria.	Cloudy, acid.	1% killed in 19 days; with antitoxin killed in 5 days.	Not recov- ered.	-
4.....	Diph- theria.	Short, granu- lar and solid rods.	+	Typical diph- theria bac- illi; branched.	Abundant on agar, serum, and potato.	Cloudy, acid.	1% killed in 48 hrs.	From fluids and organs; typical diph- theria bacilli.	-	+	Antitoxin had no effect.
5.....	Diph- theria.	Typical diph- theria bac- illi.	+	Typical diph- theria bac- illi.	Abundant on agar, serum and potato.	Clear, acid.	1% in 3-11 days; post- diphtheritic paralysis.	From seat of inoculation.	-	+
6.....	Scarlet fever and diph- theria.	Short, slender, solid rods.	-	Short rods, bipolar granules.	Abundant, yellow on all.	Clear, acid.	1% killed in 5 days; 1% in 24 hrs.	From fluids and organs.	-	+	Antitoxin had no effect.
7.....	Do	Short, solid, and long barred.	-	Same as 18 hrs. with few granular	Abundant, white on all.	Clear, acid.	1% killed in 18-24 hrs.	From fluids and organs.	-	+	Do

8.....	Normal.	Short, solid, accege-shaped rods.	-	Same as 8 hrs.	Abundant, creamy or white on all.	Cloudy, alkaline.	1% in 24 hrs.	From fluids and organs.	-	+
9.....	Measles.	Short, solid, long barred, and granular.	+	Typical diphtheria bacilli.	Like diphtheria on agar and serum; abundant on potato.	Clear, acid.	1% killed in 48 hrs.	From fluids and organs.	-	+
10.....	Scarlet fever.	Short and long solid, few barred, and granular.	+	Slender granular.	Purple on potato; turns agar purple; liquefies serum.	Clear, acid.	1% killed in 24 hrs.	From fluids and organs.	-	+	Obtained in pure culture from aural discharge.
11.....	Diphtheria.	Typical diphtheria, but small granules.	+	Typical diphtheria bacilli.	Like diphtheria.	Clear, acid.	Less than 1% killed in 30-48 hrs.	From fluids and organs.	-	+	Antitoxin had no effect.
12.....	Diphtheria.	Short and long solid, long barred.	-	Solid rods of all shapes.	Abundant, white or buff on all; gas in glucose agar.	Cloudy, acid.	1% killed in 48 hrs.	From peritoneum and organs.	-	-	From nose of case four, pure culture.
13.....	Measles.	Slender, regular, solid rods.	-	Typical diphtheria bacilli.	Abundant, white or yellow on all except serum; gas in glucose agar.	Cloudy, alkaline.	1% killed in 24 hrs.	From fluids and organs.	-	-	From same throat as No. 9.
14.....	Measles.	Short, granular, long and short barred.	-	Same as 13 hrs.	Abundant, yellow, translucent on agar and potato; gas in glucose agar.	Cloudy, acid.	1% killed in 24 hrs.	From fluids and organs.	-	-

Whenever a bacillus had proved virulent to guinea pigs, two further experiments were made with it. A dose of diphtheria antitoxin, sufficient to protect against a lethal dose of diphtheria bacilli, was given to one guinea pig together with the ascertained lethal dose of the organism in question. At the same time a second guinea pig was given a lethal dose of the organism, and also a sufficient quantity of the serum of a rabbit immunized against Ruediger's bacillus to protect against a lethal dose of the latter. The results of these experiments, as well as the cultural and morphological and chemical characteristics of the fifteen organisms, are given in Diagram 2. For the sake of brevity, the pathological changes in guinea pigs are designated as "diphtheria-like," or "like Ruediger;" the cultures on solid media are not given separately, and the description "like diphtheria" signifies grayish colonies on serum, rather scanty, translucent growth on agar, no visible growth on potato, and a delicate growth in gelatin stab not spreading and not liquefying. Derivatives from the typical *B. diphtheriæ* are entered in italics.

It is necessary to add some details to the chief features shown in the diagram:

No. 1 was from a case of typical diphtheria, but the culture, taken on one of the first days of the disease, showed only the short, solid, wedge-shaped rods usually regarded as typical pseudodiphtheria bacilli. It was only in broth cultures that the bacilli developed bipolar granules. This case confirms the statement of Westbrook that the short, solid bacillus is sometimes the only form found in cases of clinical diphtheria. At first the growth on solid media was like that of a typical *B. diphtheriæ*, but later on it became increasingly luxuriant, creamy, moist, and spreading. No. 15 of the first series was isolated from this same patient.

The virulence of bacillus No. 1 was not great: 1.5 per cent. of the body weight was required to kill a guinea pig. The changes produced were those of experimental diphtheria; the bacilli recovered were slender, barred and pointed at the ends. Diphtheria antitoxin protected guinea pigs against this bacillus.

No. 2 was from the throat of a girl who, while convalescing from measles, developed a typical scarlatinal rash, with rise of temperature and severe angina. The case was diagnosed as measles complicated with scarlet fever, and antitoxin was not administered. The tonsils and pharynx were swollen deep red, and covered with grayish patches. Cultures from the throat showed large numbers of short and long slender rods, without bars or granules although old cultures showed also short barred and granular forms. The creamy, moist, spreading growth on solid media resembled that of the Rue-

diger bacillus, and so did the effect upon guinea pigs. Doses of 1 per cent. of the body weight intraperitoneally killed in twenty-four to forty-eight hours, with lesions like those produced by Ruediger's bacillus, and the organism could be recovered from all fluids and organs. At the site of inoculation a hemorrhagic area was usually found, and in one instance, bacilli, identical in appearance (microscopic) with typical diphtheria bacilli, were found. Reference has already been made to Ohlmacher's case, in which the organisms originally isolated were simply solid, short rods, but those recovered after passage through a guinea pig were typical diphtheria bacilli.

No. 2 resembled morphologically, culturally, and in the lesions produced by it the Ruediger bacillus, but differed from it in one essential point: the diphtheria antitoxin protected animals against it, while the Ruediger serum had no protective action. Therefore, in spite of many deviations from the usual type, one is forced to consider it a member of the *B. diphtheriæ* group.

No. 3, from a case of diphtheria, resembled No. 1 in that only short, solid rods grew on serum in the first eighteen hours. The cultures resembled macroscopically those of a scantily growing diphtheria bacillus, but in its pathogenesis the organism proved not to be a strain of *B. diphtheriæ*. It was injected into two guinea pigs, one of which received also the usual dose of antitoxin, and this animal died in five days, while that which had received no antitoxin lived for nineteen days. Far from protecting, the antitoxin seemed to act unfavorably, hastening the death of the animal. The virulence of this organism was slight and transient, for when an attempt was made later on to test the action of the Ruediger serum, it was found that the bacillus no longer killed guinea pigs. No. 3 is apparently a feeble virulent pseudo-diphtheria bacillus.

The following eight belong to the group of organisms designated by Ruediger as "virulent pseudodiphtheria bacilli." They differ from each other culturally, morphologically, and in acid production, but have these two important characteristics in common: they all cause general bacteriemia in guinea pigs, and their effect on these animals is inhibited by Ruediger's serum, but not by diphtheria antitoxin.

No. 4 was from a case of pseudomembranous tonsillitis which was diagnosed clinically as diphtheria. The membrane on the tonsils and the constitutional symptoms were typical, but injections of antitoxin—2,000 units administered twice—had absolutely no effect, either locally or upon the general symptoms. The first serum cultures from the throat were not entirely typical, the rods being somewhat shorter and thicker than the usual forms. The growth on solid media was like that of a typical pseudodiphtheria bacillus; the effect upon guinea pigs was like that of the Ruediger bacillus and was neutralized by the Ruediger serum, but not by diphtheria antitoxin.

No. 4 is therefore a virulent pseudodiphtheria bacillus, and the failure of the diphtheria antitoxin to affect the clinical symptoms would seem to be explained.

No. 5 was in all respects a typical diphtheria bacillus, except that it was not neutralized by diphtheria antitoxin, but was neutralized by the Ruediger serum. Clinically this was a mild case of diphtheria. Antitoxin was administered, but it is impossible to see from the history that any effect followed.

Nos. 6 and 7 may be considered together, as, except for slight cultural differences, they resembled each other closely. They were isolated from the throats of a father and son who entered the County Hospital with the diagnosis of scarlatinal diphtheria. The father said that three other members of his family had had the same disease, one lightly, one much more severely, and the third fatally. In the case of the two in the County Hospital, the throat symptoms consisted in redness and swelling, but no membrane. The man, No. 7, had a high temperature and a vivid, "lobster-colored" rash; the boy had apparently a moderately severe case of scarlatina. Both were given antitoxin with no apparent effect. The organisms were virulent, No. 6 apparently less virulent for a man than No. 7; it was much more so for guinea pigs, proving fatal in doses as small as 0.2 per cent. of the body weight.

Nos. 6 and 7 both caused general infection in guinea pigs, both failed to be neutralized by the diphtheria antitoxin, and both were neutralized by the Ruediger serum.

No. 8 belongs to the same class as the foregoing five in pathogenesis for animals, and in the fact that it was neutralized by Ruediger's serum. Isolated from a normal throat, the organism, in morphology and cultural characteristics, was apparently a typical pseudo-diphtheria bacillus, virulent for guinea pigs, but causing no symptoms in man.

This is an additional proof that the virulence for man of a certain organism cannot be determined with entire certainty by testing it on guinea pigs. In the first series of bacilli not pathogenic for guinea pigs we have seen that four were in all probability pathogenic for man. Here we have an instance of the converse.

No. 9, from a case of measles with unusually severe throat symptoms, is the sixth organism in this series belonging to the class of virulent pseudo-diphtheria bacilli which are neutralized by the Ruediger serum. The appearance under the microscope, of this bacillus, was that of the less usual type of *B. diphtheria*, but Neisser granules were present. It grew abundantly on potato.

No. 10 was obtained in pure culture from the ear of a scarlet fever convalescent who developed an otitis media. Culturally it corresponded exactly to the organisms 16 and 17 of the non-pathogenic series, liquefying serum, causing a brownish-purple discoloration in agar slants and a growth like a smear of grape-jelly on potato. Morphologically it came nearer to the diphtheria bacillus; the rods were longer, and a few barred forms and Neisser granules were present. The virulence of this bacillus was not tested until fully five months after its isolation, when it was found to cause bacteriemia in guinea pigs, fatal within twenty-four hours. Diphtheria antitoxin had no effect. Ruediger's serum afforded full protection.

No. 11 also belongs to this class. It was isolated from the throat of a child, who, without appearing very ill, had swollen tonsils, covered with a delicate, veil-like exudate. Smears from the throat showed typical diphtheria bacilli, and 2,000 units of antitoxin were administered. At this time the child was playing about the room, but soon after the injection of antitoxin his temperature rose, he became markedly worse, and died in less than twenty-four hours. Pure cultures from the throat on Loeffler's serum showed

short and long, straight and curved, rods with Neisser granules, which, however, were of narrower diameter than the rods. On all culture media the growth resembled that of a diphtheria bacillus. Less than 1 per cent. of the body weight of a broth culture killed guinea pigs in twenty-four to forty-eight hours, producing a general bacteriemia and no change in the adrenals. Diphtheria antitoxin exerted no protective influence; indeed, the animals injected with it died somewhat earlier than those without (six to eight hours). On the other hand, the Ruediger serum did protect, so that we have here again a virulent pseudodiphtheria bacillus of the same group as Ruediger's.

Five of these eight virulent pseudodiphtheria bacilli were from cases diagnosed as diphtheria and treated with antitoxin. In two of them, Nos. 4 and 8, no alleviation of the symptoms could be seen, and in one, No. 11, the effect was apparently unfavorable. Nos. 5 and 6 were too mild for any effect to have been noted.

Nos. 12, 13, and 14 differ from the other members of the pseudodiphtheria group so far described in that they ferment glucose with the formation of gas. This property was discovered accidentally when glucose agar was substituted for the usual glycerin agar. Morphologically they might easily pass for either pseudodiphtheria bacilli or the less usual types of diphtheria bacilli, especially in older serum cultures, when typical granular forms often develop. The cultures on all media except glucose agar might pass for pseudodiphtheria bacilli. They all produced general infection in guinea pigs, but they were not neutralized either by diphtheria antitoxin or by the Ruediger serum. No. 12 was obtained in pure culture from the nose of the patient whose throat yielded No. 4 of this series. No. 13 was found in the same throat as No. 9 of this series. No. 14 was from the throat of a case of measles with severe pharyngitis.

We have, then, twenty-nine organisms which are all, with the exception perhaps of the last three, pseudodiphtheria bacilli; that is, they correspond to some member of this loosely formed group already described in the literature. They may be divided into at least three separate groups. First, there is the group of those which are non-pathogenic to guinea pigs—a group which probably should be further subdivided, for some are apparently pathogenic to man, and there are certainly decided differences as far as cultural characteristics go. Yet a basis of sub-classification would be hard to find. Second, there is a well-defined group of organisms pathogenic to guinea pigs, producing a general bacteriemia, and neutralized by the serum of a rabbit immunized against one member of the group. Third, the organisms which form gas in glucose media, produce bacteriemia in guinea pigs, and are neutralized neither by diphtheria antitoxin nor by the pseudodiphtheria serum.

The second class is much the most important, for it is apparently the most pathogenic to man, and contains those organisms which are most apt to be mistaken for the diphtheria bacillus.

Including the seven strains isolated by Dr. Ruediger, there have been fifteen organisms belonging to this group obtained during a little more than a year's time in this laboratory from clinical material which was far from abundant. The cases were scattered through the year and were not connected, except those of the family to which Nos. 6 and 7 belonged.

Five of these fifteen organisms would not have been discovered save as a result of a routine experimental study of the bacteria of a certain number of throats, for there was nothing unusual in the symptoms of the cases in question. These were, the bacillus from the normal throat, the one from the pharyngitis of measles, the one from the ear in scarlet fever, and the two from the mild cases of supposed diphtheria.

The other ten were from cases in which the clinical symptoms pointed to diphtheria, but in which the diphtheria antitoxin failed to alleviate, if it did not aggravate, the condition present.

A careful search through the literature fails to bring to light instances of diphtheria in which the injection of antitoxin proved ineffectual or deleterious, and in which a thorough bacteriological examination was made with control experiments on animals. Where such instances are mentioned—and even a passing mention is rare—it is assumed that they must have been due to “mixed infection,” the object apparently being to lay no stress on anything which might throw doubt on the absolute harmlessness of diphtheria antitoxin. Undoubtedly, many cases of fatal diphtheria and of diphtheroid pharyngitis in scarlet fever are due to the pyogenic cocci. This has been proved over and over. Yet the experience in this laboratory shows that a certain, and a not insignificant, proportion of these cases is due to invasion by a bacillus morphologically and culturally similar to *B. diphtheriæ*, but of an absolutely distinct variety.

It is a question whether in our cases the antitoxin acted more deleteriously than it often does in perfectly normal persons, when given for the purpose of immunization. Johannesen has shown

that a large proportion of normal persons react more or less severely to even small doses of horse serum. Alföldi* reports a case ending fatally on the fourth day after injection, the child having been entirely healthy at the time of the injection. In the case of the sick it is, of course, impossible to say how much the aggravation of the symptoms is due to the course of the disease itself and how much to the administration of the antitoxin. In Nos. 1 and 7 of Dr. Ruediger's cases, and in the one fatal case of my series—No. 11—there was a strong suspicion that death was hastened by the antitoxin.

It is much to be regretted that no bacteriological examination was made of the blood and organs of these fatal cases. Judging from the effect on guinea pigs, it seems probable that a general bacteriemia would be found in such cases. This, if it proves true, will lead one to ask if it is not possible that some of the instances reported as "general infection by *B. diphtheriæ*" may not belong in this class instead. Such cases have been reported by Councilmen, Wright, and others. Wright succeeded in recovering the organisms from the blood and organs of guinea pigs injected with the cultures from some of his cases. I find, however, only one report of experiments to prove the exact nature of the bacillus isolated. Stephens and Parfitt, who point out this missing link in the literature, showed that in their cases diphtheria antitoxin afforded protection to guinea pigs against a lethal dose of the organism.

In Howard's famous case of acute ulcerative endocarditis, without diphtheria, pure cultures of a diphtheria-like organism were isolated from the vegetations on the valves of the heart, and from the spleen and kidneys, but the bacillus proved to be non-pathogenic for guinea pigs.

A rapid method of diagnosis remains to be found between *B. diphtheriæ* and the virulent pseudodiphtheria bacillus—an awkward name which has nothing to recommend it but the lack of a better one. Our results confirm the experiments of Spronck, and show that animal experiments are the only sure means of distinguishing the two; but this method is obviously not adapted to a prompt diagnosis in cases where a question arises as to the administration of

* *Berl. klin. Wchnschr.*, 1895, p. 285.

antitoxin. As we have seen, morphology and presence or absence of Neisser granules are of little value as diagnostic points. The formation of acid or alkali in broth cultures is subject to too many variations to be depended upon absolutely. Schabad is perhaps the most enthusiastic advocate of this method of diagnosis, and his figures show that if the average obtained from a large number of strains of *B. diphtheriæ* is compared with the average obtained from a large number of pseudodiphtheria bacilli, the contrast is very striking. But single instances of the former may produce acid very feebly, while the latter may, during the first forty-eight hours—the time when it is most important to make a diagnosis—produce a decided amount of acid. The following results were obtained by titration of nineteen pathogenic bacilli, pseudodiphtheria bacilli of my own series and of Dr. Ruediger's, Schabad's method having been carefully followed.

Schabad gives the amount of acid produced by *B. diphtheriæ*, as follows: Two days, 5 to 22; four days, 0 to 24; seven days, 10 to 21; the numbers signifying cubic centimeters of normal sodium hydroxide solution required to neutralize one liter of broth culture.

The same method was used in the titration of broth cultures of the strains isolated by Dr. Ruediger and the pathogenic strains in my own series, with the following results:

	2 Days	4 Days
Dr. Ruediger's series		
1.....	7	1.5
2.....	7	0.5
3.....	4	2.5
4.....	2	0.5
5.....	1	1
6.....	0	1
7.....	-1	-1
My series pathogenic		
1.....	6	1.5
2.....	10	3
3.....	5	...
4.....	5	4
6.....	7	10
7.....	6	6
8.....	2	3
9.....	4	3
10.....	4	2
11.....	7	9
12.....	7	1
13.....	2	12
14.....	4	7

These figures show that, although, on the whole, the pseudodiphtheria bacillus forms less acid in forty-eight hours than *B. diphtheriæ*, yet there are strains—no less than nine in the above series—which produce as much as the less vigorous acid-producers among the former. By four days the difference is much more marked, but here, too, there are exceptions, four of the pseudodiphtheria strains still producing large quantities of acid after four days.

In point of accuracy and promptness, then, the production of acid in broth cultures fails as a method of diagnosis between the two organisms, and a more rapid and sure method is demanded. It will be my aim in continuing this study to work toward the discovery of such a method, and also to ascertain the exact nature of the immune serum obtained by injecting rabbits with cultures of these strains of the virulent pseudodiphtheria bacillus.

I take pleasure in acknowledging my indebtedness to Dr. Hektoen, not only for suggesting this investigation, but for many helpful suggestions in carrying it out.

EXPERIMENTAL STREPTOCOCCUS ARTHRITIS IN RELATION TO THE ETIOLOGY OF ACUTE ARTIC- ULAR RHEUMATISM.*

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THE following theories as to the etiology of acute articular rheumatism are held by those who believe in its bacterial origin:

1. That it is due to a specific micro-organism, as yet undiscovered.
2. That it is due to various pyogenetic cocci, mainly the ordinary streptococci; that it is a mild form of pyemia.
3. That it is due to a specific streptococcus or diplococcus.
4. That it is due to a specific bacillus—*Bacillus of Achalme*.

The evidence adduced by those holding the last view is not at all convincing. At present the second and third views have most supporters, and it is mainly these that we propose to consider.

The view that acute articular rheumatism is caused by streptococci or diplococci is based, first, on the isolation by various observers from the blood, joints, urine, or inflammatory exudates in certain cases, clinically resembling acute articular rheumatism, of streptococci or diplococci; and, second, by the production of arthritis and endocarditis in rabbits by the introduction of pure cultures of these organisms into the circulation. Furthermore, for a long time a close relationship between angina and rheumatic fever has been recognized, and Meyer has cultivated from the throats of rheumatic patients suffering from angina streptococci which when introduced into rabbits also produced arthritis and endocarditis.

Without reviewing in detail the results of the various observers who have cultivated cocci from cases of rheumatic fever, we may say that all investigators have not had similar fortunate results.

* Received for publication October 5, 1901.

With a fairly generally distributed organism like the streptococcus, the possibility of contamination must always be borne in mind, and the possibility, in the more severe cases at least, that the streptococcus infection is a terminal or secondary one, must be considered. Streptococci are so frequently found in the throat, especially in all forms of angina, that the importance of their presence in rheumatic angina must rest entirely with the demonstration of specific characteristics. So also the difficulty in differentiating acute rheumatism from certain cases of septic arthritis and acute forms of arthritis deformans adds to the chances for error.

During the past three years, in practically all cases of acute articular rheumatism treated in this hospital routine cultures have been made from the blood, and from the joints whenever any effusion was present in the latter. So far all our cultures from cases of acute articular rheumatism have been negative. Similar results have been obtained by Philip,¹ who made cultures twenty-one times from the blood and six times from the joints in twenty-four cases of acute articular rheumatism, and also made cultures twice from the blood and twice from the joints in four cases of chronic rheumatism (arthritis deformans). The cultures were made in a great variety of both solid and liquid media, and were cultivated both aërobically and anaërobically. Inoculations directly from patients were also made into guinea pigs, rabbits, dogs, monkeys, and calves. But from none of these cases could any bacteria be cultivated.

The view that the cocci hitherto cultivated from rheumatic cases are specific, *i. e.*, differ inherently from the ordinary *Streptococcus pyogenes*, is based mainly on the supposedly characteristic effects when introduced into rabbits, and to a less extent on certain cultural and morphological peculiarities. The main characteristic pathogenic effects claimed have been the development of a multiple arthritis, and in certain cases endo- and pericarditis. Meyer² laid stress on the mild character of the arthritis and the absence of bacteria in the affected joints, though other observers, making the same claim of specificity for this streptococcus, repeatedly cultivated the organisms from the inflamed

joints. However, "long ago Loeffler, with a streptococcus isolated from a case of scarlet fever angina, repeatedly produced joint inflammation, indeed so often that he definitely spoke of a 'streptococcus articulorum.' It is further quite well known that Buday produced polyarthritis in animals with streptococci isolated from a septic joint infection" (quoted by Singer³). Also Menzer, by the injection of a streptococcus isolated from a breast abscess, and of a second one isolated from a case with septic endocarditis, produced effects in rabbits similar to those which had been reported by Meyer with streptococci from rheumatic angina, and by Westphal, Wassermann, and Malkoff⁴ with streptococci from a case of chorea, and by Menzer and others from rheumatic fever cases. The evidence up to 1902 in regard to the bacterial etiology of rheumatic fever has been all collected and reviewed by Menzer,⁵ and I have, therefore, not detailed the previous observations. Menzer concluded:

The cause of acute articular rheumatism is not a specific bacterium, but the ordinary parasites of the mouth, which under certain conditions have become pathogenic. Almost exclusively are streptococci the organisms concerned. These streptococci are by far the most common cause of the different forms of angina, and also of the so-called angina rheumatica.

Since the publication of Menzer's monograph, however, several English observers* have continued to report the isolation and study of a diplococcus or streptococcus from the blood, joints, urine, or inflammatory exudates of certain cases clinically resembling acute rheumatic fever. This organism they regard as a specific micro-organism, and consider as a cause (some of them as *the* cause) of rheumatic fever, and for it Walker has proposed the name *Micrococcus rheumaticus*. Poynton and Paine have not claimed the discovery of this organism, but in their earlier papers have endeavored to show its identity with the organisms described previously by Triboulet, Dana, and Westphal, Wassermann and Malkoff. As the present paper proposes to deal with the claim as to the specificity of the organisms described, it will not be necessary here to give the details as to the manner of isolation and source of the organisms studied.

* Poynton and Paine, Ainley Walker, Beaton and Walker, Shaw, Beattie. (See list of references at the end of this paper.)

The claims of the various writers as to specificity of the organisms isolated are based on morphological, cultural, and pathogenic peculiarities of the organism isolated. In the various papers published, reports, usually quite meager, of the morphological and cultural characteristics are given. But in none of them have I been able to find any definite characters given which will serve definitely to identify this organism from the ordinary *Streptococcus pyogenes*. In the first reports it was spoken of as a diplococcus, then as a diplococcus frequently growing in chains, and in some of the papers very long chains are described.

To summarize briefly the results obtained in the efforts to separate this coccus on morphological or cultural grounds, we cannot do better than quote certain conclusions from the papers of Beaton and Walker and of Shaw, whose papers (except that of Beattie, which adds nothing to this aspect of the subject) have appeared last and deal more extensively with the cultural and morphological characteristics than the others have done. Walker says:

This organism has been classed by Paine and Poynton as a diplococcus; by the German workers it is usually called a streptococcus. So far as our own observations go, the pairing of the cocci is usually very marked in recent cultures, and more especially in the original cultivations, and in body fluids or on agar-agar; and chains may be quite infrequent in the films. Under cultivation the arrangement in chains becomes more marked, and cultures grown in milk or in a highly alkaline bouillon frequently present long chains, which in some cases may extend across one or more fields of the microscope with a one-twelfth oil immersion objective. When growing in the tissues—as, for example, in endocarditic vegetations in the human subject—the majority of the cocci are seen to be in pairs, but sections not infrequently show chains of half a dozen individuals or more.

Whether the micrococcus be spoken of as a diplococcus or a streptococcus does not appear to be of any great importance, and might be regarded merely as a matter of words.

But we think that the persistent application of the former term to an organism which is at present indistinguishable on culture media from an ordinary streptococcus, and which in cultures often shows a marked tendency to grow out into chains, has probably tended to delay considerably its general recognition by bacteriologists: for we have no hesitation in saying that *without animal experiments it would almost certainly be diagnosed as an ordinary streptococcus by anyone* [italics ours] to whom cultures and stained preparations were submitted without comment.

Culturally this organism resembles a streptococcus, but that it is specifically different from the ordinary streptococci is rendered probable by our results on the application of the test of Marmorek.

Since, however, the value of this test for determining specificity has had so much doubt thrown upon it by the work of Meyer,¹¹ and Aronson,¹² and others, and especially since Walker's observations were so few and reported with such slight detail, this evidence at present, at least, cannot be considered very valuable.

Shaw's results are especially important in this connection, as he worked with organisms obtained from several observers. He describes the organism as a "streptococcus, and, though usually smaller than the *Streptococcus pyogenes* commonly met with, *it might easily be mistaken for it* [italics ours]. It grows well on the ordinary nutrient media." The only characteristic given which might serve to separate it from *Streptococcus pyogenes* is the following: When grown on blood agar "the blood agar undergoes a change in color. The bright red color is replaced by a dull brown or rusty appearance, or even a greenish-brown tint." He says: "In the case of several other varieties of micrococci investigated, this alteration in color does not occur, *e. g.*, with the *Streptococcus pyogenes* and the *Streptococcus septicæmiæ*" (?).

Reference to a publication of Schottmüller's,¹³ however, shows that a similar reaction has been described as occurring with a large number of streptococci obtained from widely differing sources, none of which were rheumatic fever cases. These races have been grouped by that writer into a separate variety, to which he gives the name *Streptococcus milior seu viridans*, in contra-distinction to the variety *Streptococcus longus pathog. seu erysipelatos*, by which only hemolysis of the blood medium is produced, but no discoloration. He calls special attention to the fact that in the cases where the former variety occurred the infection was milder than that associated with the latter variety.

It is further stated by Shaw that

the characteristics described above apply, in the first place, to the organism isolated by Wassermann and Westphal, but they apply, without modification, as far as morphology and cultural characteristics go, to the micrococcus kindly sent me by Dr. E. W. A. Walker from Guy's Hospital, and to that sent by Dr. F. J. Poynton from the Hospital for Sick Children, Great Ormond Street.

From all this it is evident that at present the claims for specificity of this organism must rest on the results obtained by inoculation into animals.

Practically all of the observers who have been mentioned as describing a coccus associated with rheumatic fever, have been able, by the inoculation into rabbits of quite large amounts of this streptococcus, to induce multiple arthritis of variable grades of intensity, in some cases endocarditis and pericarditis, and in a few experiments a condition said by the writers to resemble chorea. It would lead us too far to attempt a detailed description and report of the experiments of these observers whose papers are all easily accessible.

WRITER'S OWN EXPERIMENTS.

While making a series of cultures from cases of acute rheumatic fever and endocarditis, a streptococcus was cultivated from the blood of the patient whose history is given below in abstract.

STREPTOCOCCUS FROM CASE "RAND."

BARBER, COLORED, AGED FIFTY. ADMITTED NOVEMBER 12, 1903.

The patient said he had always been quite well up to five or six years before admission, when he began to have "heart trouble." For a year or more he had been troubled with "rheumatic pains in his joints," though no definite history of acute rheumatic fever could be obtained.

Following a spree four weeks before admission, the patient noticed swelling of both legs, right hand, and arm, and he complained of headache and pains in his joints. He soon became slightly irrational. This lasted a few days, and then he became suddenly unconscious, and it was found there was complete right-sided hemiplegia. The right arm was constantly in motion with spasmodic movements. The mental condition began to clear up on the third day, and there was gradual improvement in the muscular power. On November 10 there came another sudden attack of unconsciousness, and again complete loss of power on right side.

On admission he apparently understood what was said to him, but could not articulate distinctly. There was almost complete right-sided flaccid hemiplegia. The area of cardiac dullness was moderately increased. The heart action was very irregular, and there were signs of aortic and mitral insufficiency. There was no edema of extremities, and no definite signs of arthritis could be made out. Temperature, 101°5; pulse 100.

The patient remained in about the same condition, the temperature being continually elevated, ranging between 100° and 103°, until November 17, when a blood culture was taken and a streptococcus isolated, the number of colonies averaging about 6 to 1 c.c. of blood. The patient's condition gradually grew worse, and death occurred on December 7. At no time was any arthritis made out. The clinical diagnosis was acute endocarditis, cerebral embolism, septicemia. Cultures from the blood were made, as above stated, on November 17, and again on November 20, on the latter date about 30 colonies developing from each cubic centimeter of blood. On November 24 cultures were

again made from the blood, about thirty-five colonies to each cubic centimeter developing, and on November 30 blood cultures again showed streptococci in about the same number. On December 6 blood cultures were again made, with a like result.

Autopsy showed as the principal lesions acute and subacute vegetative mitral and aortic endocarditis, cardiac hypertrophy and dilatation, chronic passive congestion of the internal viscera, multiple infarction of spleen and kidneys, thrombosis of left middle cerebral artery, with extensive softening of the internal capsule.

Although this organism grew well on ordinary media, and culturally no difference from the common *Streptococcus pyogenes* could be made out, it was thought, owing to the presence of endocarditis and history of a previous attack of rheumatism, that possibly the organism was identical with the so-called "*Micrococcus rheumaticus*;" so a series of inoculation experiments was undertaken. The study of this organism formed the beginning of the present series of observations.

As the organism grew rather better on glycerin agar than on plain agar, and as most of the inoculation experiments reported by the English observers had been made with emulsions made of the growth on dry media, either blood agar or glycerin agar, it was thought best to continue the use of this medium and method for the inoculation material. Slant 6 per cent. glycerin agar tubes were used, and the inoculating material was thoroughly smeared over the surface with a platinum needle. After twenty-four hours' growth about 1 c.c. of bouillon was poured over each tube, and the growth scraped off with a platinum needle and thoroughly mixed with the bouillon. After the desired number of tubes of suspension had been so prepared, they were poured together into a sterile dish, and the fluid drawn into a glass syringe and in all cases inoculated into the ear vein, being careful that it all entered the vein and did not spread subcutaneously. In this way ear abscesses were avoided almost entirely.

INOCULATION EXPERIMENTS.

Rabbit I.—Rabbit apparently healthy. Temperature, 99°6.

XI-28: Inoculation into the ear vein of an emulsion made from growth on four glycerin agar tubes (twenty-four hours' growth).

XI-29: Rabbit apparently well.

XI-30: Lame in left front leg: will not put left foot to ground; hops about on three legs; the left elbow is swollen and sensitive. Temperature, 101°8.

XII-1: Some improvement in the left fore leg, but there appeared to be some soreness in the left hind leg. Temperature, 102°4.

XII-2: Left hind leg still seems tender. Temperature, 100°8.

XII-3: Condition better, no lameness. Temperature, 99°6. Reinoculated; emulsion made from growth on five glycerin agar tubes (twenty-four hours' growth). There was no lameness or swelling of the joints following this inoculation; temperature ranged between 99°4 and 99°8, and animal did not appear sick until XII-9, when the temperature was 101°, and the rabbit was quite lame in the left hind leg. The lameness persisted until XII-13, when death occurred. The autopsy showed slight reddening of the inner

surface of the capsule of the left hip joint and a small amount of slightly turbid, thick, tenacious, exudate in the joint. There was no roughening of the cartilage. A similar condition was found in the left elbow. All the other joints appeared normal. There were no microscopic lesions in any of the viscera. Smears were made of the exudate in the left hip and elbow. These were stained with an aqueous solution of methylene blue. From both joints the cellular constituents of the exudate were found to be mainly polymorphonuclear cells, with a few large mononuclear cells. A few biscuit-shaped diplococci were found in smears from each joint.

The upper part of the left femur, with as much of the capsule of the joint as possible attached, was hardened in alcohol and decalcified in a nitric acid solution. Longitudinal sections through this piece show a mild inflammation of the superficial layers of the inner surface of the capsule, especially marked at the edge of the cartilage and just outside this line. The surface is covered by a small amount of exudate containing mostly polymorphonuclear leucocytes and a few red blood corpuscles. The cartilage covering the head of the bone is quite smooth and unaffected.

Unfortunately the records from cultures of this rabbit have been lost.

Rabbit II.—Rabbit apparently healthy. Temperature, 99°4.

XII-9: Inoculation into the ear vein of an emulsion made from the growth on six glycerin agar tubes (twenty-four hours' growth).

The rabbit appeared to be stiff in its movements several days after the inoculation, though at no time could any definite lameness or swelling of the joints be made out. The temperature ranged between 100° and 102° from XII-12 to XII-20, on which day the animal died.

At autopsy all of the joints were carefully examined, but no macroscopical lesions were found in any except the left shoulder, where the inner surface of the capsule showed considerable redness, and there was a small amount of tenacious, sticky, mucopurulent exudate. No erosion of the cartilage was seen. The internal organs appeared normal. Cultures made from the heart's blood, liver, kidney, spleen, gall bladder, right shoulder, left elbow, and right knee showed no growth. The culture from the left shoulder showed a growth of streptococci which were identified as identical with those inoculated.

Smears made from the exudate in the left shoulder joint showed many polymorphonuclear leucocytes and some large mononuclear cells. A moderate number of diplococci were seen. These were usually oval with the long axis transverse to the direction of the pair and the adjacent surfaces slightly flattened. No chains were found.

The left shoulder, with portion of the humerus and scapula, was hardened, decalcified, sectioned, and stained. The sections show a considerable infiltration of the capsule, and swelling and infiltration of the villous projections. There is no erosion or roughening of the cartilage. The infiltration is mainly with polymorphonuclear cells.

Rabbit III.—This rabbit had two inoculations, the first on XII-17 of an emulsion from six glycerin agar tubes (twenty-four hours' growth), and again on XII-29 of an emulsion from the growth on six glycerin agar tubes, the organism used for the latter growth being that obtained from the joint of Rabbit II. After the first inoculation no lameness was made out, but after

the second inoculation, on XII-31, there were definite tenderness and lameness in the right fore leg and in the left hind leg. No swelling of the joints could be detected.

The autopsy showed a mild involvement of the right shoulder, left hip, and right hip, similar to that seen in Rabbits I and II.

Cultures made from the heart's blood, left knee, left hip, left elbow, right hip, right knee, and right elbow were all sterile. On the plate made from the right shoulder there developed about twenty colonies, all of which were alike, and which were proved to be streptococci like those inoculated.

Although there was no growth from the right hip, smears of the exudate made from this joint showed a few diplococci. No diplococci, however, could be made out in smears from the left hip, though the exudate was quite purulent, more so than that in the right hip.

Sections were made through both hips and show quite marked infiltration of the villi and capsule near its junction with the cartilage with small round cells and polymorphonuclear leucocytes. There is considerable congestion apparent about the joint, and the exudate into the joint shows many pus cells, as well as mononuclear cells. The left hip shows more marked changes than the right.

Rabbit IV.—XII-29: Inoculation into the ear vein of an emulsion made from the growth on six glycerin agar tubes (twenty-four hours' growth). The organism in this case was that obtained from the left shoulder of Rabbit II.

On the third day following the inoculation the rabbit began to appear sick, would not move unless urged, and all its joints appeared painful; the animal would not use the left hind leg. This lameness and stiffness continued, at times one extremity appearing more lame, and again another. The animal lost rapidly in weight. The lameness, however, grew less, and on I-11 it was inoculated intravenously with an emulsion made from the growth on six glycerin agar tubes (twenty-four hours' growth) of organism from Rabbit II. On I-14 the lameness and stiffness in joints again began, especially marked in the left hind leg. This again disappeared by I-19. On this day a similar reinoculation was made as on previous occasions. On this occasion the lameness and stiffness were not evident until a week after the inoculation. Again, while the lameness was quite general, it was most marked in the left knee. This lameness lasted three days. Following this, the rabbit was inoculated on I-29, II-16, III-1, III-29, and IV-7, on each occasion lameness and stiffness of the joints appearing four to seven days after the inoculation. As, however, the lameness and joint involvement became less with the later inoculation, it was thought well to try the effect of a different strain, so on IV-12 inoculation with the organism obtained from the case "Gold" was made. The results with autopsy are given below (case "Gold," Rabbit IV).

Rabbit V.—This animal was repeatedly inoculated with an emulsion from the growth on glycerin agar tubes of organisms from Case "Rand." The inoculations occurred on I-11, I-19, I-28, II-1, II-10, III-2, III-29, IV-7, IV-23, and IV-27.

From four days to a week after each one of the inoculations there were very definite tenderness and lameness in the larger joints. There is still marked lameness in the left fore leg three weeks after the last inoculation. The animal has not yet been killed.

Rabbit VI.—I-29: Inoculated intravenously with an emulsion made from the growth on six glycerin agar tubes (twenty-four hours' growth). The organism was obtained from the right shoulder of Rabbit III. There was no evident effect, and on II-4 a second similar inoculation was made. One week following this there were swelling and tenderness of the left shoulder. The animal was quite lame, would not put left fore foot to the ground. The right hind leg also seemed stiff and painful. On II-17 another inoculation similar to the first two was made. Three days later the swelling and lameness of the joints again became very marked. This lameness lasted for several days.

III-1: Another similar inoculation was made with similar results, and the rabbit died on III-7.

At autopsy it was found that the right shoulder contained an excess of sticky, slightly turbid fluid. The inner surface of the capsule was injected. There was no erosion of the cartilage. Similar slight changes were found in the left shoulder and both hips. The other joints all appeared normal on macroscopical examination. The internal organs showed no gross macroscopical lesions.

Cultures made from the left knee, left elbow, and left hip showed the growth of a moderate number of streptococci, corresponding in all characteristics to those inoculated. Cultures from the right knee were sterile. From the heart's blood, liver, and right kidney there was a very profuse growth of streptococci. Coverslips made from the heart's blood, liver, and kidney showed a moderate number of diplococci, while in those made from the left shoulder no cocci could be found after a prolonged search. Unfortunately, smears were not made from the other joints.

Sections were made from the head of the left femur, but practically no changes could be made out.

Finding that the results obtained by inoculation of this streptococcus agreed quite closely with those obtained by other observers with inoculation of the so-called "*Micrococcus rheumaticus*," it was decided to try streptococci from other sources in a similar manner. The following protocols give the results obtained with six other races of streptococci from various sources.

STREPTOCOCCUS NO. 2223.

This was obtained at autopsy from the peritoneal cavity of a man, aged fifty-seven, dying from an acute terminal infection complicating carcinoma of the stomach. There was no arthritis or endocarditis, and no history was obtained of rheumatism.

INOCULATION EXPERIMENTS.

Rabbit I.—Inoculated I-19-'04, intravenously. The emulsion was made from the growth on four glycerin agar tubes (twenty-four hours' growth). No lameness or swelling of the joints was made out before death, which occurred after forty-eight hours. At autopsy there were no macroscopical lesions in any joint. A pure culture of streptococcus was obtained from the heart's

blood. No cultures were made from the joints. The organism obtained from the heart's blood in this case was repeatedly transferred on glycerin agar and then inoculated into another rabbit.

Rabbit II.—Inoculated IV-18-'04, intravenously. The emulsion was made from the growth on seven glycerin agar tubes (twenty four hours' growth). The organism was obtained from Rabbit I.

On the third day after inoculation the rabbit was stiff and lame in both hind legs. There was considerable swelling of the left tarsal joint. This tenderness and swelling of the joints were more marked on the fourth day, when death occurred.

Autopsy.—The right knee, both hips, and both elbows appeared quite normal on gross examination. The tissues about both tarsal joints were very edematous, and the joints contained an excess of turbid fluid. Both shoulders also contained an excess of turbid fluid, the joint capsules being distended and reddened. The exudate was not so sticky as seen in some of the other cases. The cartilage appeared unaffected. The left knee was also distended with turbid fluid; no erosion of the cartilage. No gross lesions were made out in the internal organs, except in the heart. Both segments of the initial valve showed considerable thickening along the free margins. One of the segments was removed and sections made. These show a typical acute valvular endocarditis. There is marked thickening and infiltration with polymorphonuclear leucocytes and, in places, minute ulcers are seen.

Cultures were made from the heart's blood, spleen, liver, kidney, right tarsus, right and left shoulders, right knee. In all cultures there was a moderately profuse growth of streptococci. In smears made from the heart's blood no cocci were found. A few diplococci were present in smears made from the spleen and liver, and they were very numerous in the kidney smears. In coverslips made from the left knee very numerous diplococci were found; no chains were seen. In the coverslips made from both shoulders and left ankle a great many diplococci were found, and these were sometimes collected into short chains of three to five pairs.

STREPTOCOCCUS NO. 2228.

This streptococcus was also obtained at autopsy from a case of puerperal septicemia with acute endocarditis and pericarditis. Death occurred three weeks after abortion, previous to which time the patient had been quite well. There was no arthritis. Streptococci were obtained from the heart's blood during life and the clinical features of the case were those of septicemia.

INOCULATION EXPERIMENTS.

Rabbit I.—Inoculated I-23-'04, intravenously, the emulsion being made from the growth on two glycerin agar tubes (forty-eight hours' growth). On the third day there were noted some lameness and stiffness in the right hind leg. This continued for two days. On I-29-'04 the animal was reinoculated with the emulsion from four glycerin agar tubes, and again on II-4-'04 with

the emulsion made from six tubes (twenty-four hours' growth). Three to four days after each inoculation the tenderness and lameness became more marked. With the last inoculation the lameness was most marked in the left fore leg.

On II-9-'04 the rabbit was killed. At autopsy the tissues about the left shoulder were found to be very edematous and swollen. The joint was much distended, and the capsule was filled with a purulent fluid containing flaky masses. The right knee joint was also found to be affected. On opening the capsule of the joint a sticky, purulent fluid exuded. The capsule was found reddened, especially along its junction with the cartilage. The right shoulder also showed a mild inflammatory reaction. The right elbow was hardened *in toto* without opening. The other joints all appeared normal. The internal organs showed no gross lesions.

Cultures made from the heart's blood, liver, spleen, kidney, right elbow, left knee, right knee, and right shoulder showed no growth whatever. On the plates made from the left shoulder there occurred quite a profuse growth of streptococci, resembling those inoculated. Three loops of the fluid were inoculated in each plate, and on each about fifty colonies developed. Coverslips made from the exudate in the left shoulder showed a moderate number of diplococci; no chains could be found. It was impossible to find any diplococci or other bacteria in coverslips made from any of the other joints. Sections were made from the right shoulder, left shoulder, right knee, and right elbow. The left shoulder showed the most marked changes. There is an abundant purulent exudate, the cellular elements of which are mainly polymorphonuclear cells, but scattered among which are seen many larger mononuclear cells. The tissues about the joint also show a marked polymorphonuclear infiltration, and there are everywhere very marked edema and congestion of the blood vessels. Notwithstanding the marked inflammatory reaction, however, the free surface of the cartilage is perfectly smooth. The right shoulder shows a milder infection, though there is a considerable inflammatory exudate, containing many polymorphonuclear leucocytes, with thickening and infiltration of the capsule, especially of the villus projections about the edges of the joint. The right elbow also shows quite a marked inflammatory reaction of the same nature as seen in the shoulders. As this joint was not opened before hardening, the increased exudate into the joint is very well shown. The tissues outside the joint do not show such marked changes as were seen in the left shoulder. The right knee shows the slightest changes of all, though even here the congestion and infiltration of the villi is very well marked.

Rabbit II.—Inoculated with emulsion made from growth on four glycerin agar tubes of organism obtained from the joint of Rabbit I. Death occurred in twelve hours. Joints normal.

Rabbit III.—Inoculated III-2-'04, intravenously. The emulsion was made from growth on three glycerin agar tubes of organisms obtained from joint of Rabbit I. Two days later the rabbit was very lame in the left fore leg, and it seemed sick and not inclined to move about. This lameness and apparent weakness and sickness continued until III-7-'04, when the animal began making inco-ordinated movements, throwing itself about in the cage and, when placed on the floor, running about violently in various directions,

apparently not seeing where it was going and running its head into the walls. Respiratory movements were very jerky and rapid. The animal died in the afternoon.

Autopsy.—Joints: The muscles and tissues about the left shoulder joint appeared very edematous. On opening the joint the capsule was found to be distended with a moderate amount of a yellowish, purulent, cloudy fluid. The cartilage appeared normal. The inner surface of the capsule was injected. The right shoulder joint resembled the left. The capsules of the left knee and of both hips were also slightly injected, and in these joints there was a slight increase in the amount of fluid which was slightly cloudy. The other joints appeared normal.

Heart: There was quite marked dilatation, especially of the right side. The tricuspid and pulmonary valves appeared normal. The aortic valves also appeared thin and normal. Adhering to the edge of one of the cups of the mitral valve was a long clot which, on attempting to remove it, was found to be a thrombus attached to a rough area on the valve. This area was covered by small vegetations.

The liver was congested. The spleen appeared normal. The left kidney was larger than the right and contained numerous small abscesses the size of a pinhead. The pelvis of the left kidney was injected and distended, and contained a purulent fluid. The urine was very cloudy, and contained a large amount of sediment. The bladder mucosa was injected.

Cultures made from both shoulders showed numerous streptococci. There was also a growth of a few streptococci from the right hip. No growth, however, occurred in the cultures from the left knee and right elbow. Also there was no growth in the cultures made from the heart's blood. Streptococci also grew in the cultures from the liver, spleen, and right kidney. Cultures made from the urinary bladder and left kidney showed a pure growth of *B. coli communis*. It therefore seems probable that the abscesses in the left kidney and the urinary infection were due to a secondary infection, possibly local in origin.

Coverslips made from the exudate in both shoulders show a moderate number of diplococci. No chains were found. No bacteria could be demonstrated in smears made from either hip. Smears from the right kidney showed a moderate number of diplococci, while in smears from the urine only short rods could be discovered. No bacteria could be demonstrated in coverslips from the heart's blood or liver. The left shoulder joint was removed entire and decalcified, and sections were made. The joint contained a moderately purulent exudate, and there was a moderate grade of infiltration of the capsule with polymorphonuclear cells. The cartilage appeared normal. Sections through the left kidney showed numerous very small abscesses.

(This case illustrates well the occurrence of an endocarditis with involuntary, inco-ordinated movements, which by a stretch of the imagination might be called choreiform.)

Rabbit IV.—This rabbit was inoculated intravenously on IV-8-'04 with an emulsion made from the growth on four glycerin agar tubes of the streptococcus obtained from the joint of Rabbit III. Three days later the animal was very lame in the right hind leg. The stiffness extended to the other

joints, but the right knee remained most affected and was considerably swollen. On IV-15-'04 the rabbit was killed, and an autopsy was performed immediately. On opening the right knee joint it was found to contain an excess of slightly turbid, sticky, mucoid, fluid, containing some small grayish flakes. The cartilage was smooth, but the lining of the capsule was hyperæmic. The left elbow also contained an excess of a similar fluid, and the capsule presented the same appearance. The right shoulder joint appearing distended with fluid, only a small puncture was made in order to obtain a culture, and the whole joint was then hardened unopened. The other joints appeared normal. There were no signs of endocarditis, and the other organs appeared normal, except the kidneys. These were pale, and showed on section quite numerous small pin-point areas which were grayish and resembled minute abscesses. Sections made from these kidneys show quite numerous small focal areas of infiltration with small round cells. The capsule is much thickened, and there is a new growth of connective tissue extending some distance below the surface. Practically no polymorphonuclear infiltration is seen anywhere.

Cultures were made from the internal organs and joints. No growth occurred in cultures from the heart's blood, spleen, liver, or kidney. The cultures from the left shoulder, left knee, and right elbow were also sterile, while in the cultures from the right knee, right shoulder, and left elbow there developed a considerable number of streptococcus colonies.

STREPTOCOCCUS OBTAINED FROM CASE "M'CABE."

This was a case of cardiac hypertrophy and dilatation in a man aged thirty-one. He had been a stoker and worked very hard. No other etiological factor could be obtained. He had never had rheumatism, any form of arthritis, or any acute infection. The patient was admitted to the hospital on I-25, and from this time until III-5 he suffered with severe symptoms of cardiac insufficiency, but there was no fever. On II-5, however, the temperature suddenly rose, and he began having high fever, sweating, and delirium. Blood cultures on III-7 showed the presence of streptococci in large numbers, 140 colonies to 1 c.c. of blood. The patient died on III-8. At autopsy marked sclerosis of the coronary vessels was found with fibrous myocarditis, but there was no acute or chronic endocarditis. In addition, there were infarction of the lungs and acute pleurisy.

INOCULATION EXPERIMENTS WITH ORGANISM OBTAINED FROM BLOOD DURING LIFE.

Rabbit I.—Inoculated III-29-'04, intravenously, with emulsion made from twenty-four-hour growth on six glycerin agar tubes. On the following day the rabbit was found to be quite lame in the right fore leg. This lameness increased, and the tenderness gradually spread to the other joints. The

animal could not be induced to move about, and passive movements of the joints seemed to cause pain. No evident swelling in any of the joints could be made out. Three days after inoculation the animal began to show general convulsive twitchings of all the extremities. These convulsive movements increased up to the time of death, which occurred on the following day.

Autopsy.—The pericardium contained a moderate increase of slightly turbid, bloody fluid. The endocardium was slightly blood-stained; no endocarditis made out. No abscesses were found in the liver, spleen, kidneys, or lungs. The tissues about the right knee were slightly edematous. The lining of the capsule was reddened and covered with a thick, sticky, turbid fluid. The capsule of the joint was moderately distended. There was also some edema about the left knee. The entire joint was hardened unopened. Both elbows and the left knee showed changes similar to those seen in the right knee. The involvement of the right tarsus was greater than that of the other joints, the edema of the tissues about being very marked and the exudate quite purulent. No changes could be detected in the right hip, and in the left hip there was merely slight reddening of the lining of the joint, and a moderate increase of sticky, slightly turbid fluid.

Sections were made from the elbows, knees, shoulders, and tarsal joints, the left knee and right elbow being hardened unopened. In all the joints examined there was found a mucopurulent exudate, with a slight infiltration of the capsule and synovial membrane with polymorphonuclear leucocytes, the most marked changes being noted near the folding of the membrane upon itself about the bone, while the cartilage in all cases was quite smooth and appeared normal.

Coverslips made from the pericardium showed quite numerous diplococci and short chains of two or three pairs of cocci. In those made from the heart's blood, liver, and kidneys diplococci were found in considerable numbers, and also a few short chains consisting of three or four pairs of diplococci; but no long chains were found. In coverslips made from both elbows, both tarsal joints, right shoulder, right knee, and left hip, cocci were found, though in variable number, very many being present in the joints most involved, while in those less involved fewer were present. In all cases the cocci were in the form of diplococci, occasionally short chains of two or three pairs being seen. In all cases there were numerous polymorphonuclear cells, though small mononuclear cells were also present in considerable numbers. The cocci were in all cases extra-cellular.

Cultures from the heart's blood, kidney, and liver, showed typical streptococci growing in broth into long chains. There occurred also a moderately profuse growth on plates made from the left tarsal joints, left hip, right knee, and right shoulder. In all cases the growth was a pure one of typical streptococci growing into long chains.

STREPTOCOCCUS FROM CASE "GOLD."

This case was one of empyema, probably following an acute lobar pneumonia, though the onset was rather insidious. There had never been any rheumatism or arthritis. The heart sounds

were clear. On aspiration of the chest turbid, purulent fluid was obtained, cultures from which gave a pure growth of streptococci. The chest was later freely opened, drainage established, and the patient made a complete recovery.

INOCULATION EXPERIMENTS.

Rabbit I.—This rabbit died twenty-four hours after inoculation intravenously with an emulsion made from the twenty-four-hour growth on five glycerin agar tubes. At autopsy no joint involvement could be made out, though coverslips from the right knee showed a few puss cells, and a considerable number of diplococci and short chains of cocci. Cultures and coverslips from the internal organs all showed streptococci.

Rabbit II.—This rabbit died twelve hours after inoculation with an emulsion made from the twenty-four-hour growth on three glycerin agar tubes. No changes in any joints were found at autopsy. Cultures showed a general septicemia, but cultures from the joints were negative.

Rabbit III.—Inoculated IV-23-'04 with an emulsion made from the twenty-four-hour growth on two glycerin agar tubes. The animal did not appear very sick following this injection, until the fourth day when the lameness was quite marked. Both fore legs and the right hind leg seemed affected. The animal moved about with difficulty. This condition of stiffness and lameness persisted and increased until IV-30-'04, seven days after inoculation, when death occurred.

Autopsy showed no endocarditis and no purulent foci in any of the internal organs. Both knees appeared normal. There was considerable edema about both tarsal joints, and the capsules were reddened and distended with sticky, turbid fluid. The right hip showed a slight turbid exudate and reddening of the capsule. The left elbow and both shoulders also showed a similar mild involvement. The left hip and right elbow appeared normal. Both shoulder joints were hardened entire. Sections through these showed the joint moderately distended with a muco-purulent exudate and an infiltration of the capsule. In the left shoulder the cartilage was somewhat roughened and irregular, but whether this was due to the inflammation or to manipulation could not be determined.

Coverslips from the kidney and spleen showed a moderate number of diplococci. In the heart's blood no cocci could be definitely detected. In coverslips from the right hip, the right ankle, and the left knee diplococci were present in considerable numbers; no chains could be found. In the left ankle the diplococci were in much smaller numbers, and a few short chains of two and three pairs were found.

Cultures made from the spleen and kidney showed a moderately profuse growth of streptococci. The cultures from the liver and heart's blood showed a very scant growth of streptococci. They were present in cultures from the left knee, right tarsus, and right shoulder in moderate numbers; much fewer in the left ankle, while in cultures made from the right knee, right elbow, and left shoulder no growth occurred.

Rabbit IV.—This is the same rabbit as Rabbit IV under those inocu-

lated with organism "Rand." After repeated inoculation with this organism and repeated attacks of lameness, it was thought that a moderate grade of immunity had been established, so that it was inoculated with the organism obtained from the case "Gold" which appeared more virulent. Inoculated IV-12-'04 with emulsion made from growth on two glycerin agar tubes. After three days the animal did not appear lame or sick. It was again inoculated with an emulsion made from the growth on four glycerin agar tubes. Four days later the rabbit died. During life no swelling of the joints was detected: the animal did not appear lame.

Autopsy.—No purulent foci in the heart, lungs, liver, spleen, or kidneys. The right testicle was found to be swollen, the capsule red and hemorrhagic. No area of softening or abscess formation could be made out on section. Joints: The right knee was larger than the left. The joint contained an excess of thick, sticky, turbid fluid, while the capsule was reddened and showed minute areas of injection, but the cartilage appeared normal. The left knee had a similar appearance, though was not so swollen as the right. No changes were made out in either hip. The elbows, left shoulder, and left tarsus also appeared normal. The right shoulder and right ankle showed changes similar to those seen in the knees.

Coverslips made from the heart's blood, liver, spleen, and kidney all showed diplococci. A few very short chains of two or three pairs were also seen. In coverslips from both knees and from the right shoulder numerous diplococci were found. In coverslips from the left shoulder the diplococci, while present, were very few in number. In cultures made from the heart's blood, liver, kidney, spleen, and testicle there occurred a pure growth of streptococci. There occurred also a pure growth in the cultures from the knees and from the right shoulder: in these, however, the number of colonies was much less than in cultures from the internal organ.

Sections were made from the right knee and right shoulder. These show, in addition to the acute changes seen in the other rabbits, very marked thickening of the villous outgrowths and an infiltration of them with polymorphonuclear leucocytes. There is marked endarteritis of the vessels of these villi. The capsule is thickened, and in places the cartilage covering the end of the bone is much eroded and has become fibrous and infiltrated. The right testicle shows a diffuse acute inflammation and foci where the polymorphonuclear infiltration is more marked, but without true abscess formation.

STREPTOCOCCUS OBTAINED FROM CASE "D."

This was a case of acute, apparently uncomplicated appendicitis. Appendectomy was performed, following which the patient continued to have high fever, sweating, and the clinical features of septicemia. There were no signs of peritonitis; there was no arthritis. Blood cultures showed the presence of streptococcus. The patient died. Permission for autopsy could not be obtained.

* INOCULATION EXPERIMENTS WITH STREPTOCOCCUS OBTAINED FROM BLOOD.

Rabbit I.—Innocolated IV-6-'04 with emulsion made from the twenty-four-hour growth on six glycerin agar tubes. Three days later the animal became lame in the left fore leg, and it also showed some general stiffness of all extremities. There was gradual improvement of this condition, and on IV-12-'04 a second inoculation was made of the growth on three glycerin agar tubes. As following this the animal continued to show no symptoms, a third inoculation of the growth on four glycerin agar tubes was made on IV-15-'04, and a fourth one of the growth on six glycerin agar tubes on IV-16-'04. Two days later the rabbit appeared very lame on the left hind leg. The lameness increased, the swelling of the left tarsus became more marked, and on IV-21-'04 the rabbit was killed.

Autopsy.—No focal lesions could be made out in any of the viscera. All the joints appeared normal, except the left shoulder and the left tarsus. These joints showed exudate and capsular inflammation, exactly as seen in the other rabbits.

Coverslips made from heart's blood, spleen, liver, and kidney were negative. Neither were any cocci found in the coverslips made from the right shoulder and right knee. In those made from the left shoulder, however, a very few diplococci were found. Cultures made from the heart's blood, liver, spleen, and kidney, and from both knees, were also negative, but in the cultures from the left shoulder a moderate growth of streptococci occurred.

STREPTOCOCCUS OBTAINED FROM CASE "L."

This was a child aged three years, suffering with tuberculosis of the tibia. The knee joint was not involved. Operation was performed, and the child was doing well when it developed scarlet fever, contracted apparently from another child in the ward. Following the acute symptoms of the scarlet fever there developed an acute cervical adenitis. The glands were incised, and from the purulent contents cultures were made which showed a pure growth of streptococci.

INOCULATION EXPERIMENTS.

Rabbit I.—On IV-28-'04 this rabbit was inoculated intravenously with an emulsion made from the twenty-four-hour growth on three glycerin agar tubes. Death occurred twenty hours after inoculation. No distinct changes could be made out in any of the joints. Cultures made from the various organs showed streptococci, and they also grew in cultures made from both knee joints.

Rabbit II.—This rabbit was inoculated V-4-'04 with an emulsion made from the twenty four-hour growth on one glycerin agar tube. No lameness that could be detected resulted, and death occurred after forty-eight hours. No gross lesions could be detected in this rabbit. Both knees appeared a little injected, but the changes were not definite.

Coverslips showed diplococci, and short chains in the internal organs and

also in both knees. Streptococci were also cultivated from both knees as well as from the internal organs.

Rabbit III.—On V-16-'04 this rabbit was inoculated with one-fourth of an emulsion made from the growth on one glycerin agar tube. After two days there was quite evident lameness in the left hind leg. This lameness continued, there appeared general stiffness, and on V-21-'04, or five days after inoculation, death occurred. No macroscopic focal lesions were found in any of the internal viscera. The joints were all carefully examined, and no changes could be made out in any of them except the right tarsus, both hips, and both shoulders. These showed a moderate distension with turbid fluid, injection of the capsule, etc., exactly as was seen in the other rabbits. This was also well shown in the microscopical examination of the sections made through the left shoulder. Coverslips made from the heart's blood of this rabbit showed quite long chains of cocci, some of them composed of twenty to thirty cocci. (This was the only case in which this was noted.) Coverslips from the affected joints, however, showed only diplococci. Cultures made from the heart's blood, liver, and kidney showed quite a profuse growth of streptococci. Streptococci in smaller numbers also grew from the right shoulder, right knee, and left hip. No growth occurred in the cultures from the left knee. In the cultures made from this case, in addition to the streptococci, there also grew from the heart's blood, liver, kidney, and right shoulder a few large colonies, which were identified as *B. coli*. As they occurred in such small number in only a part of the cultures, and the autopsy was not performed for some hours after death, it is probable that this was a post-mortem invasion or a contamination.

It will thus be seen that experiments were undertaken with six races of streptococci aside from those with the race obtained from a possible rheumatic endocarditis Case "Rand." The sources of these various races were: peritonitis following carcinoma of the stomach; puerperal fever-autopsy; blood during life in terminal septicemia following myocarditis; empyema; blood during life in septicemia following appendicitis; scarlet fever adenitis.

The diversity in the sources of these races renders it impossible that they could all have had any association with rheumatism, so that we could not have been working always with the so-called "*Micrococcus rheumaticus*."

Our experience, as shown by the protocols, shows no essential difference in the results obtained between the several races studied, except in the virulence; that is, the amount necessary to produce the desired results. So, too, a careful comparison of our results with those obtained by Poynton and Paine, and others, fails to show any essential differences. The essential point claimed for

the characterization of the specific coccus causing rheumatism is its ability to produce arthritis and endocarditis when inoculated intravenously into rabbits.

With all seven races studied intravenous inoculation of amounts insufficient to cause death of the animals within six or seven days led to the production of mild grades of arthritis. The only reason we have to offer why this arthritis has not previously been noted by all observers who have done experimental work with streptococci is that usually such work is done with the idea of testing the pathogenicity of organisms, usually determined by the amount and time necessary to produce a fatal result, and when post-mortem examinations were made the joints have not been carefully studied as a routine procedure. During life a rabbit having arthritis will usually remain quiet in one corner of the cage, and it is only when the rabbit is placed on the open floor and urged to move about that the lameness is detected. So, too, in most cases the swelling of the joint, unless extreme, is rather difficult to detect through the thick skin and fur. Often a considerable distension of the joint and edema of the surrounding tissues has been found after dissecting away the skin, when previously it could not with certainty be detected. In many cases the joint affection is mild, and unless one were especially interested in looking for it, it might be overlooked. It is only by making a routine examination of all joints that the lesions are discovered.

From the work given it seems that, when streptococci from whatever source are inoculated intravenously into a rabbit, they tend early to localize in the joints, even before any definite lesions can be detected. In Rabbit I, Group Case "Gold," twenty-four hours after inoculation; Rabbit I, Group Case "L," twenty hours after inoculation; and in Rabbit II, Group Case "L," forty-eight hours after inoculation, streptococci could be found in the joints with no evidence of inflammation. In another rabbit inoculated with streptococci from a case of meningitis, death occurred thirty-six hours after inoculation, and in both knees, which macroscopically appeared normal, streptococci were found on cover-slips and cultures. On the other hand, in one rabbit—Rabbit II, Group Case "Gold"—dying twelve hours after inoculation, no organisms

could be grown from the joints. It is therefore probable that after intravenous inoculation with streptococci these organisms very soon are found in the joints, and finding here a *locus minoris resistentiae*, or conditions favorable for their growth, may persist here and set up a mild grade of infection. In certain cases the organisms may disappear from all the other parts of the body, and remain in the joints, as in Rabbit IV, "Streptococcus No. 2228," where one week after infection no organisms could be cultivated from any of the internal organs, while they readily grew from certain joints.

In most cases the joint affection set up is mild and tends to recovery. This tendency to recover is well seen in Rabbit IV, Group "Rand," in which for four months arthritis was repeatedly induced, and just as often recovery took place.

In most cases the pathological changes in the joints are mild, consisting mainly of edema about the joint, moderate increase of fluid within the joint, the fluid becoming turbid, sticky, and tenacious; injection of the capsule and slight infiltration, mainly of the villi, with polymorphonuclear leucocytes. In only one or two joints did the appearance resemble that seen in severe septic arthritis. It must be said that the features were more like those seen in acute articular rheumatism, though in the cases which I have seen of articular rheumatism with exudate into the joints the exudate has always been quite thin and fluid, though frequently clotting quickly after removal.

Endocarditis.—In two of the rabbits a true endocarditis was present. One of them was inoculated with streptococcus from the peritonitis case, the other from the puerperal fever case. Section through the valves in both of these cases showed a typical acute valvular endocarditis. In several of the other rabbits there was slight thickening of the valves noted, which at first was thought possibly due to endocarditis, but on section they were found to be merely slight thickenings, too old to have been due to the inoculations. In both of the cases the mitral valve was the one affected, and the involvement consisted of small vegetations along the edge of closure. While in this series of inoculation experiments endocarditis did not occur so frequently as in some of the series

reported by English observers, yet the fact that typical endocarditis occurred with two of the organisms studied, and that this endocarditis was identical with that described by the other observers, makes a differentiation of the organism on this ground untenable.

Chorea.—Several observers have claimed to have produced chorea in rabbits by the intravenous injection of the “*Micrococcus rheumaticus*.” I have not been able to find any complete description by Poynton and Paine of the symptoms resembling chorea produced by them, though they repeatedly refer to having produced chorea in rabbits. Beattie describes one rabbit in which on the second day after inoculation “there was very definite chorea, with twitching of the head and eyes to the right side. When the animal attempted to walk it fell to the right side and the twitching became much more marked.” Two days later “the chorea was much less marked,” and on the following day “the twitchings had practically ceased.”

Referring to the protocols previously given, it will be seen that Rabbit I, Group “McCabe,” and Rabbit III, Group No. 2228, showed similar convulsive inco-ordinate movements before death. Most clinicians, however, would doubt the diagnosis in a case of chorea lasting two or three days, and I should hardly like to say that the features in *my* rabbits at least resembled even remotely a case of chorea. In Beattie’s case, from the description of movements, always in one direction, one would think it more probable that a focal abscess or a localized meningitis had been present.

It may be stated that, while slight morphological and cultural differences existed between the various races of streptococci studied, none of these were constant enough to speak of a distinct variety. Each one differed slightly from the other in its manner of growth in bouillon, its growth in milk, etc. To make such comparative studies of any value, they should be repeated at intervals over a considerable length of time, to make certain that the characteristics have at least a fair degree of constancy. This has not yet been done with the organisms studied. So far as length of chain is concerned, corresponding with the observations of many others, we could determine no constant character, as this property seemed to depend much more on the character and reaction of the

medium used than on the race of the organism. It is worth while, however, referring again to the fact that in smears made from the joints, and also to a considerable degree from the organs of rabbits inoculated with streptococci, long chains are almost never met with, the cocci appearing as diplococci or as very short chains composed of a few diplococci, and in these pairs the individual cocci are usually somewhat flattened on one side, the flat sides approximated, causing the cocci to have a long diameter transverse to the length of the chain. It may be that this fact is responsible for the so-called organism of rheumatism having sometimes been called a diplococcus and again a streptococcus by various observers.

It will be seen from the protocols that there was considerable variation in the pathogenicity as measured by the lethal dose of the races studied. Thus, while with certain of the organisms an emulsion made from the growth on six to eight tubes could be safely inoculated, in the case of the streptococcus obtained from the cervical adenitis in scarlet fever, one-fourth of a tube caused death in five days when so inoculated. It is of interest that this race from a local lesion should have been so much more pathogenic than those obtained from general infections.

CONCLUSIONS.

Arthritis and endocarditis may be produced by the intravenous inoculation of rabbits with streptococci from various sources, and the results obtained are quite similar to those described as resulting from the inoculation of the so-called "Micrococcus" or "Diplococcus rheumaticus."

Therefore the description of a distinct variety or species of streptococci based on this property of causing endocarditis and arthritis is unwarranted. Whether the evidence is sufficient that acute rheumatic fever is simply a form of streptococcus septicemia it is not intended to discuss in this paper.

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CORRIGENDA.

On pp. 112-126, the chemical formula $\text{NA}_2\text{C}_2\text{H}_4\text{O}_6$ should read $\text{NA}_2\text{C}_4\text{H}_4\text{O}_6$ throughout the paper.

P. 231, third line beneath the diagram: instead of "lobular" read "lobar."

P. 341, line 2: instead of "their" read "this."

P. 521, line 15: instead of "Plate III" read "Plate XIII."

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[illegible]

PLATE I.



FIG. 1.



FIG 2.

PLATE II.



FIG. 1.

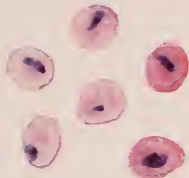


FIG. 2.



FIG. 3.

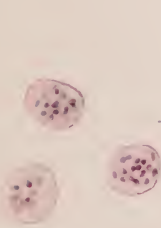


FIG. 4.



FIG. 5.

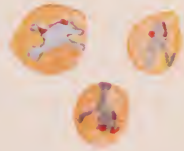


FIG. 6.



FIG. 7.



FIG. 8.

PLATE II. SHOWING PYROPLASMA HOMINIS (CAMERA LUCIDA SKETCHES).

FIG. 1.—Blood from finger, Case 107, treated with iodimized gum acacia.

FIG. 2.—Blood from circulation, Cases 89, 94, and 107. Stained eosin-methylene blue. Selected cells.

FIG. 3.—Blood from circulation, Case 125. Stained Nocard's method. Selected cells.

FIG. 4.—Blood from circulation, Case 120. Stained Nocht-Romanowsky's method. Selected cells.

FIG. 5.—Blood from circulation, (a) Case 120; (b) Case 94; (c) Case 89. Stained eosin-methylene blue. Selected paired forms, two of which are extra-cellular.

FIG. 6.—Blood from circulation, Case 125. Stained Nocard's method. Selected cells showing amoeboid forms.

FIG. 7.—Phagocytes which have taken up infected r. b. c., Case 94: (a) liver; (b) kidney. Stained eosin-methylene blue.

FIG. 8.—Phagocytes which have taken up infected r. b. c.: (a) spleen, Case 94; (b) blood from circulation, Case 125. Stained Nocard's method.

PLATE III.



FIG. 1.

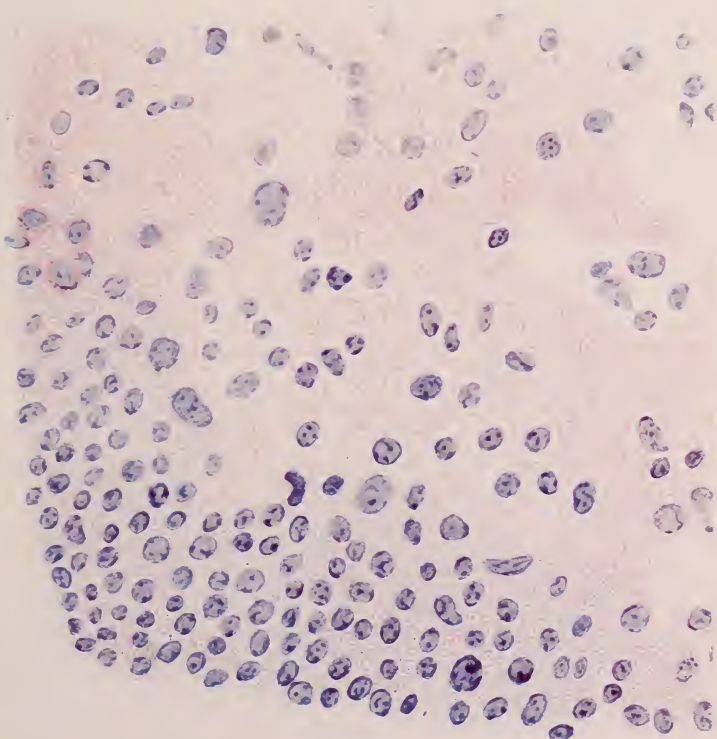


FIG. 2.

PLATE IV.

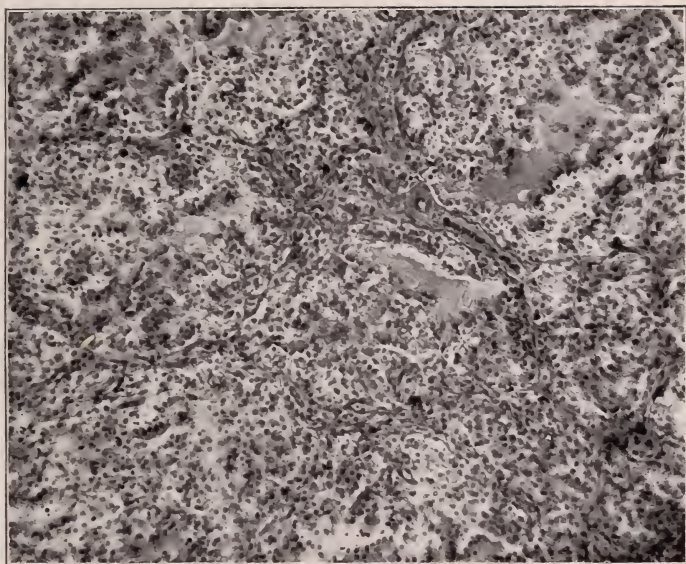


FIG. 1.

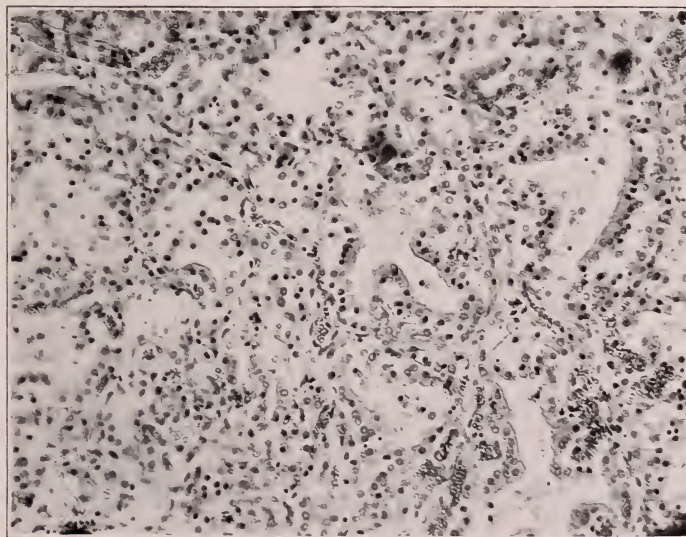


FIG. 2.

PLATE V.

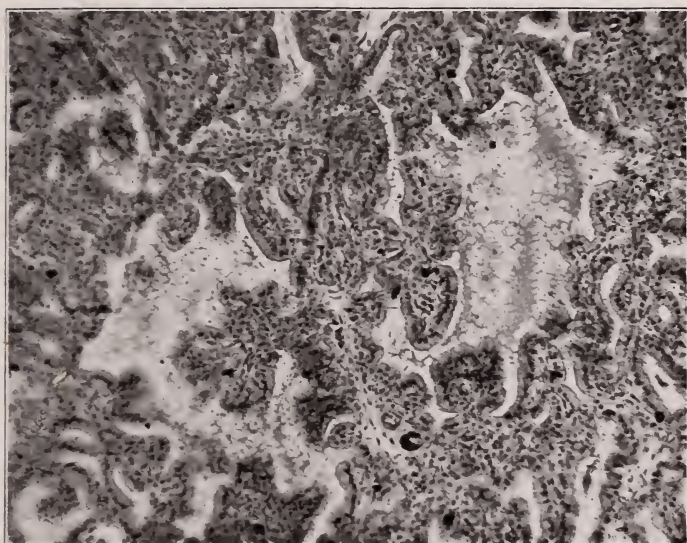


FIG. 3.

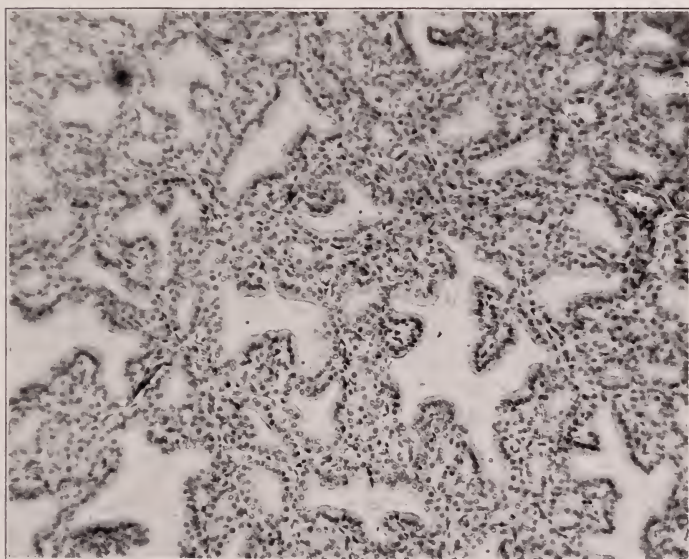


FIG. 4.



FIG. 1.

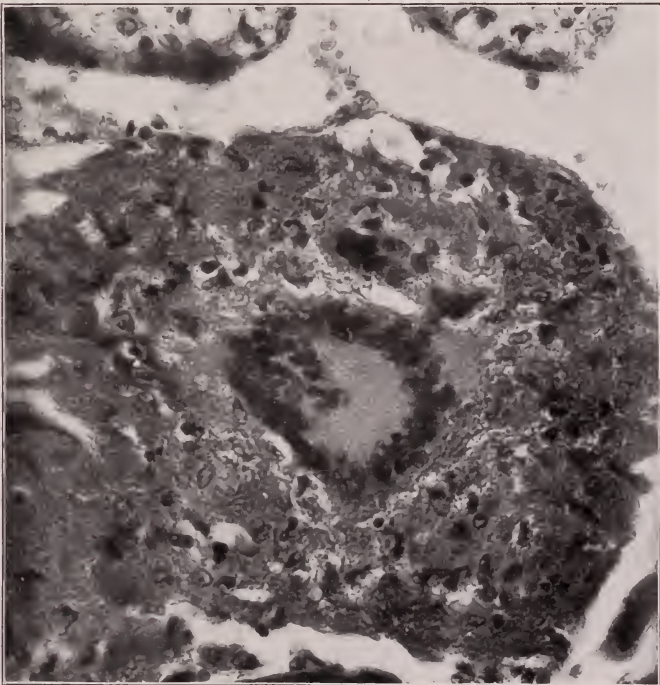


FIG. 2.

PLATE VII.

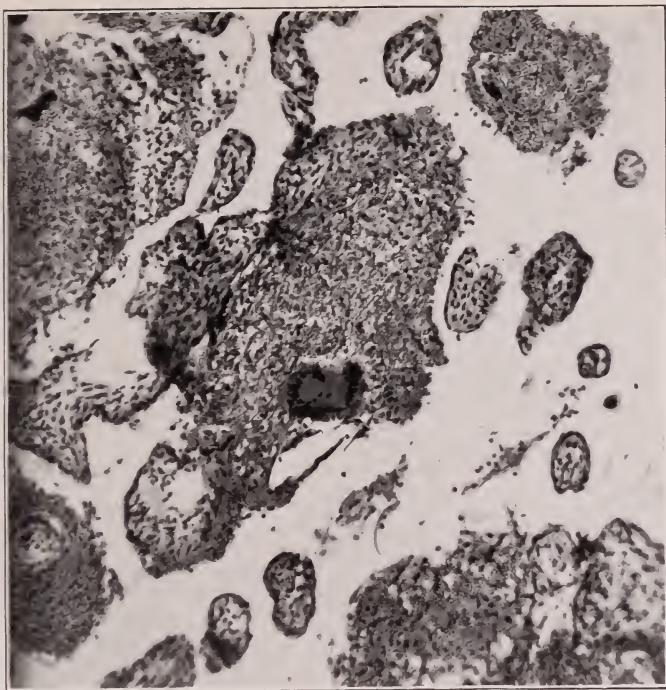


FIG. 3.

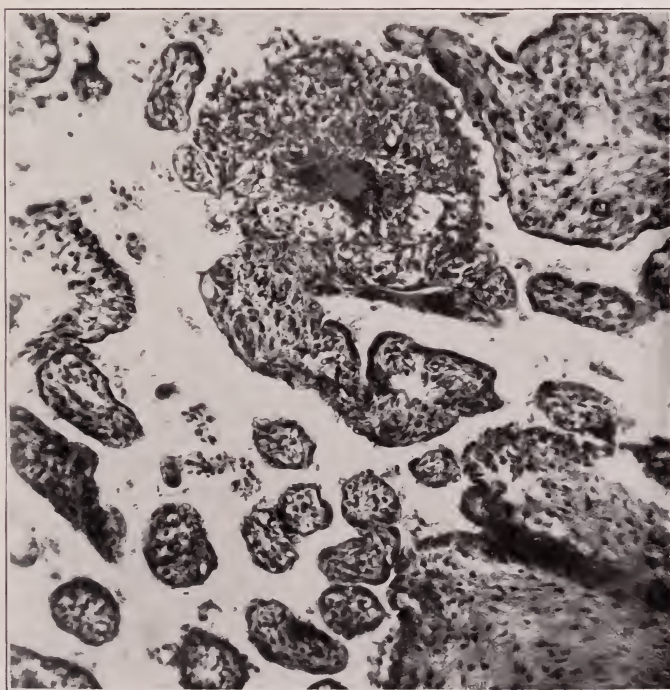


FIG. 4.

PLATE VIII.

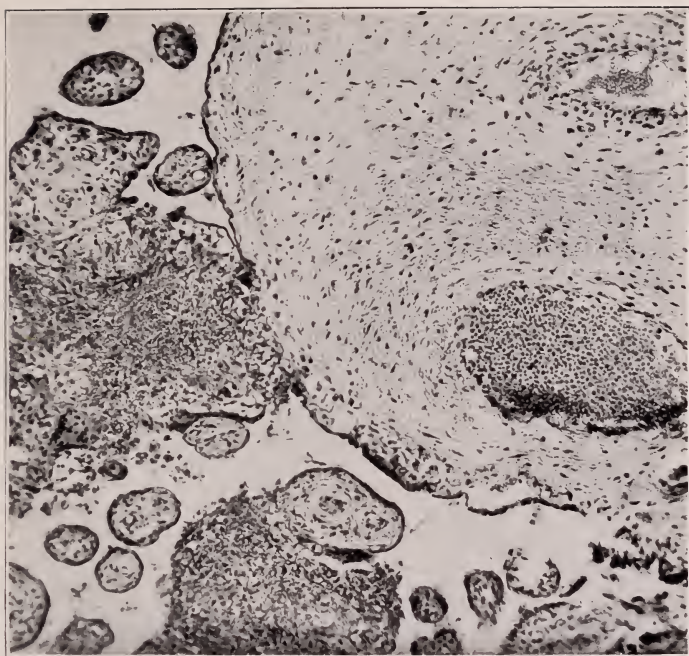


FIG. 5.

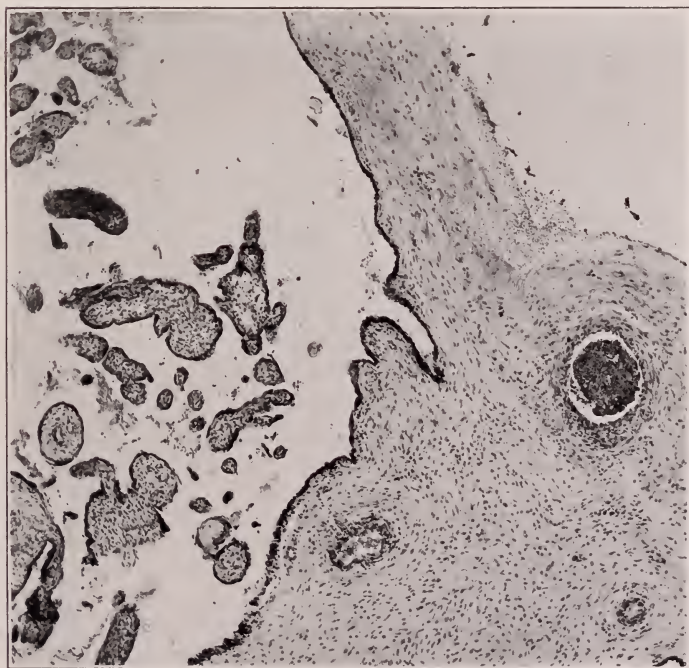


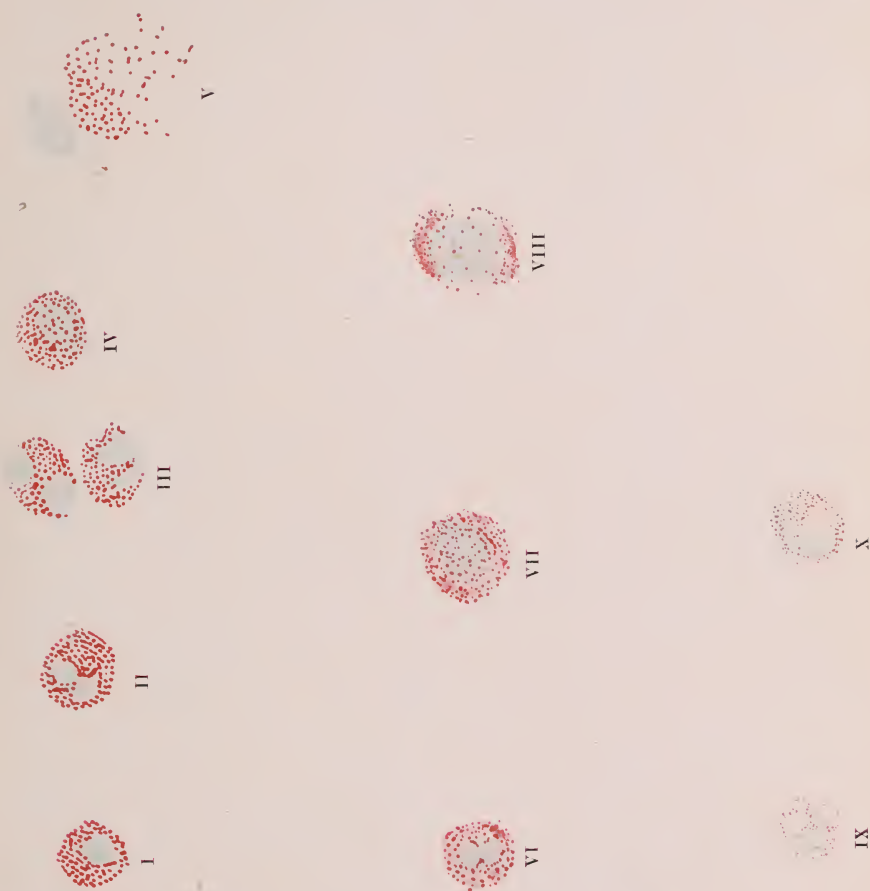
FIG. 6.

PLATE IX.



Typical reaction of the *B. coli* in lactose bouillon in the fermentation tube, showing the yellow-red reaction with neutral red.

PLATE X.



Nos. I-V, eosinophiles from favorable cases of moderate scarlatina; Nos. I-IV, showing coarse granules crowding the cells; No. V, ruptured cell; Nos. VI-VIII, eosinophiles from malignant scarlatina, oxyphile granules having washed appearance; Nos. IX, X, polymorphonuclears with active oxyphile characters.

PLATE XI.



FIG. 1.



FIG. 2.



FIG. 3.



FIG. 4.



FIG. 5.



FIG. 6.



FIG. 7.



FIG. 8.



FIG. 9.

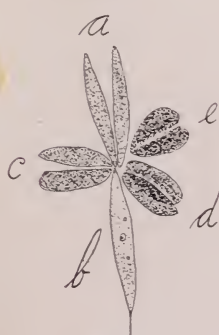


FIG. 10.



FIG. 11.

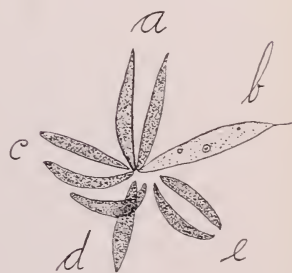


FIG. 12.

PLATE XII.



FIG. 1.



FIG. 2.



FIG. 3.



FIG. 4.

PLATE XIII.



FIG. 1.

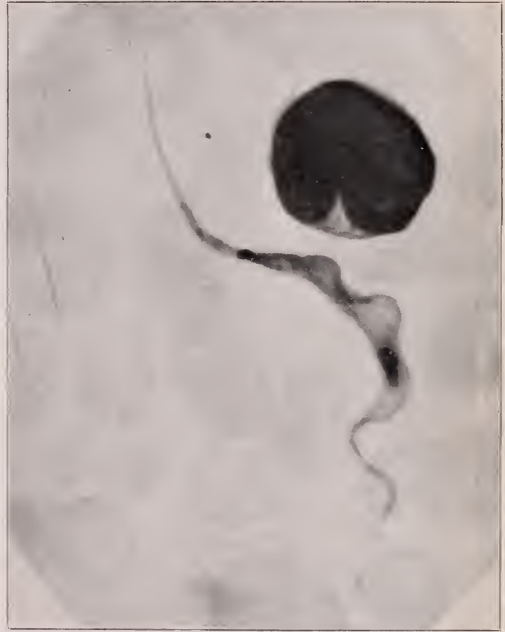


FIG. 2.



FIG. 3.



FIG. 4.

PLATE XIV.

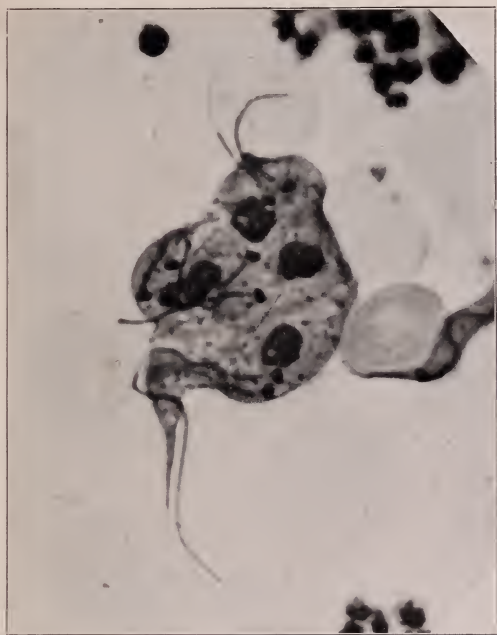


FIG. 1.



FIG. 2.



FIG. 3.

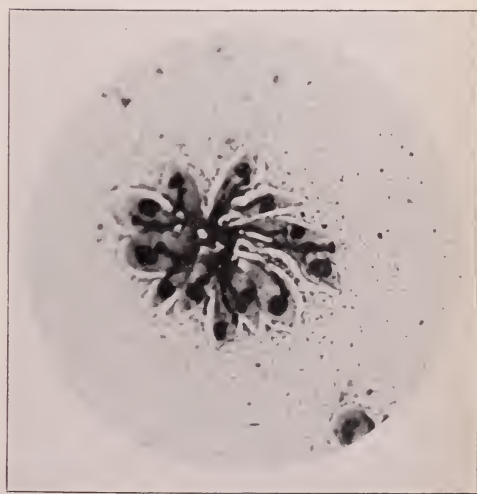


FIG. 4.

PLATE XV.

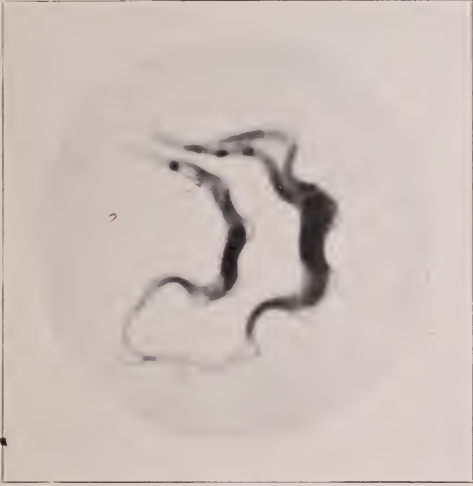


FIG. 1.

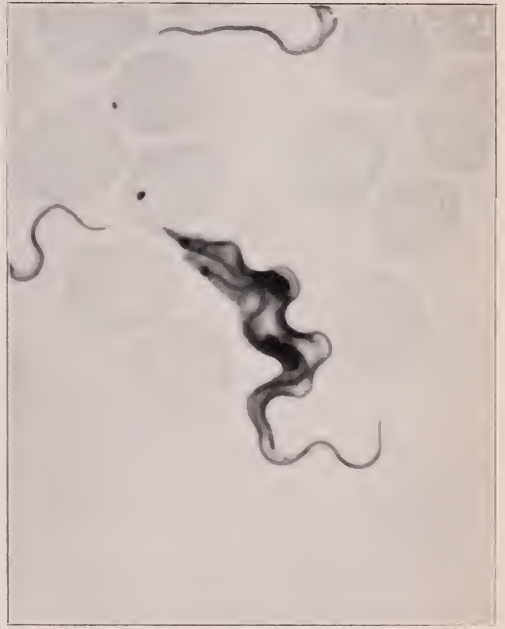


FIG. 2.

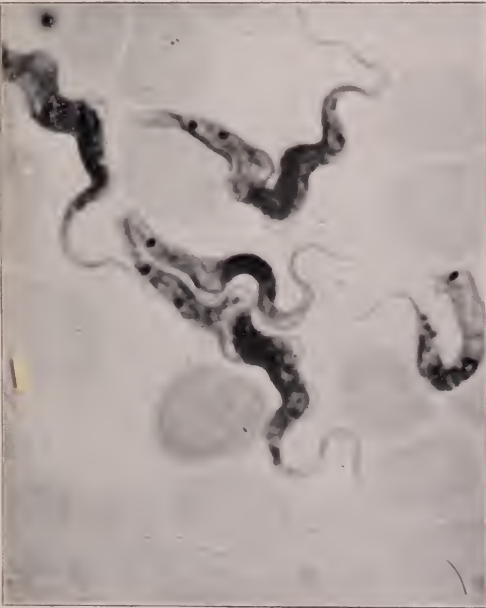


FIG. 3.



FIG. 4.

PLATE XVI.

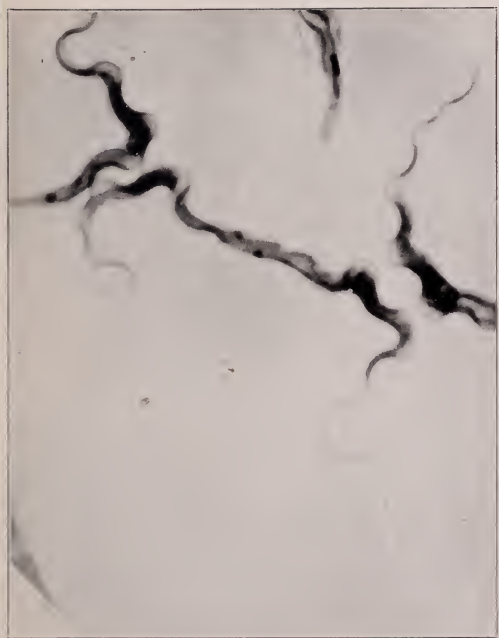


FIG. 1.

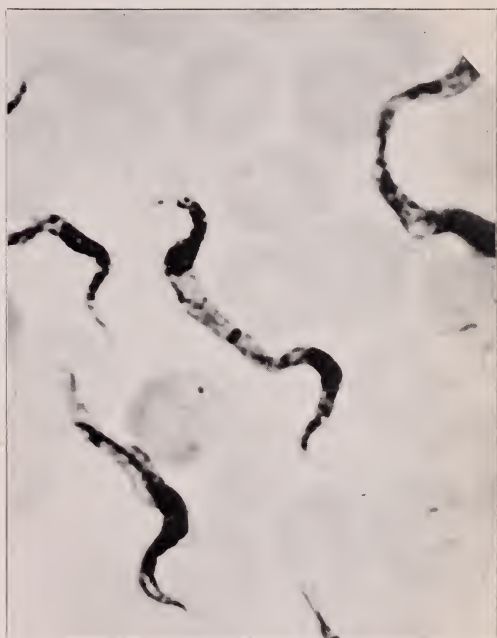


FIG. 2.

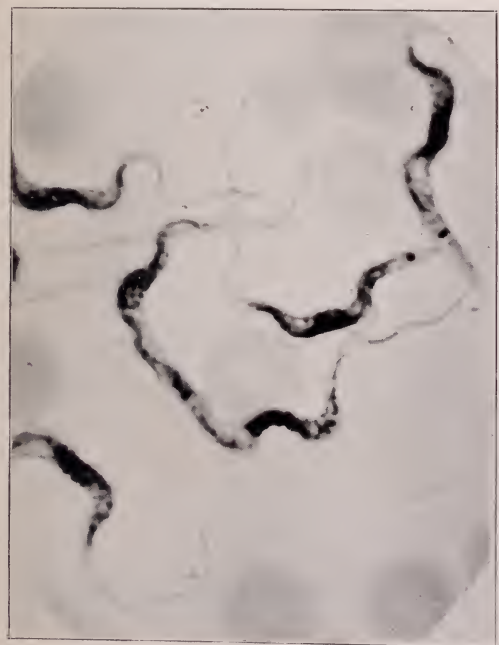


FIG. 3.



FIG. 4.

PLATE XVII.

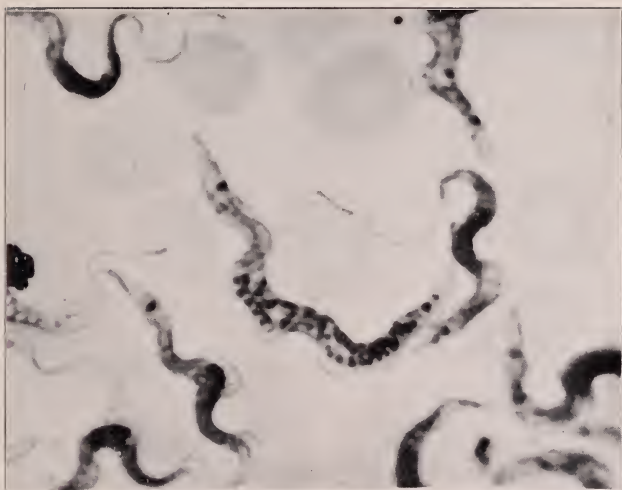


FIG. 1.

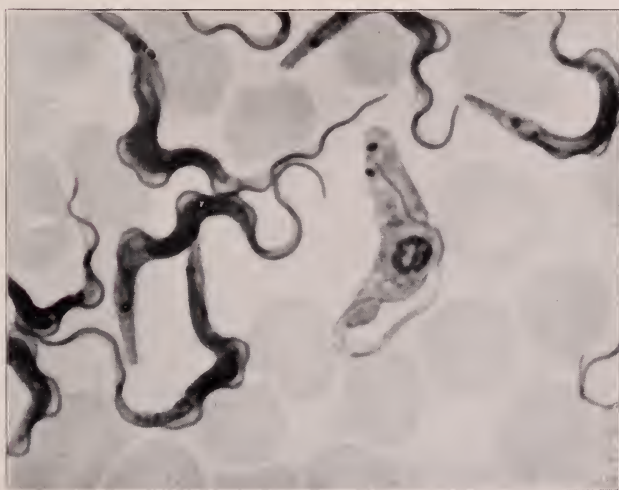


FIG. 2.

PLATE XVIII.

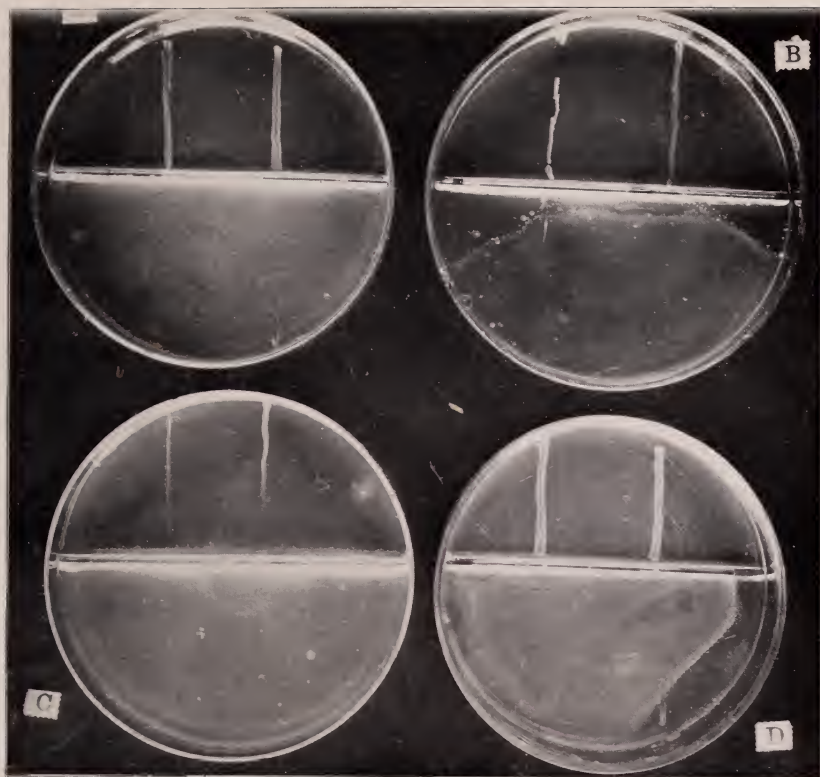


FIG. 1.

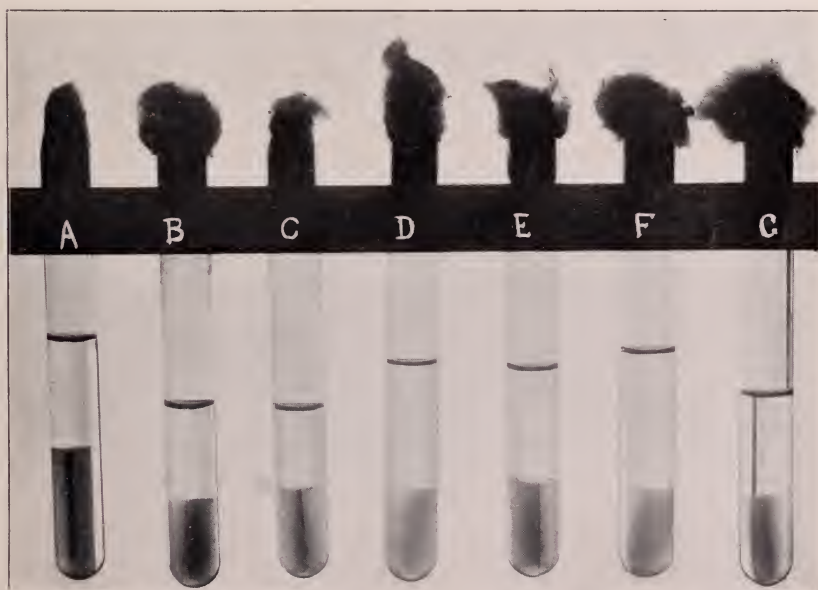


FIG. 2.

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